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ORIGINAL PAPER

Lysophosphatidic acid rescues bone mesenchymal stem cells from hydrogen peroxide-induced apoptosis

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Abstract The increase of reactive oxygen species in infracted heart significantly reduces the survival of donor mesenchymal stem cells, thereby attenuating the therapeutic efficacy for myocardial infarction. In our previous study, we demonstrated that lysophosphatidic acid (LPA) protects bone marrow-derived mesenchymal stem cells (BMSCs) against hypoxia and serum deprivation-induced apoptosis. However, whether LPA protects BMSCs from H_2O_2-induced apoptosis was not examined. In this study, we report that H_2O_2 induces rat BMSC apoptosis whereas LPA pre-treatment effectively protects BMSCs from H_2O_2-induced apoptosis. LPA protection of BMSC from the induced apoptosis is mediated mostly through LPA_3 receptor. Furthermore, we found that membrane G protein G_{i2} and G_{i3} are involved in LPA-elicited anti-apoptotic effects through activation of ERK1/2- and PI3 K-pathways. Additionally, H_2O_2 increases levels of type II of light chain 3B (LC3B II), an autophagy marker, and H_2O_2-induced autophagy thus protected BMSCs from apoptosis. LPA further increases the expression of LC3B II in the presence of H_2O_2. In contrast, autophagy flux inhibitor bafilomycin A1 has no effect on LPA's protection of BMSC from H_2O_2-induced apoptosis. Taken together, our data suggest that LPA rescues H_2O_2-induced apoptosis mainly by interacting with G_i-coupled LPA_3, resulting activation of the ERK1/2- and PI3 K/AKT-pathways and inhibition caspase-3 cleavage, and LPA protection of BMSCs against the apoptosis is independent of it induced autophagy.

Keywords Bone marrow-mesenchymal stem cells (BMSCs) · Lysophosphatidic acid (LPA) · Hydrogen peroxide (H_2O_2) · Apoptosis · Autophagy

Introduction

Cardiovascular disease is one of the most prevalent diseases with high fatality rate in modern life. Ischemic heart disease is the major cause of myocardial infarction and heart failure. This is because that most cardiomyocytes suffer to apoptosis and death from stresses of hypoxia, nutrition starvation and oxidative stress. Recent studies report that bone marrow-derived mesenchymal cells (BMSCs) display distinct properties in self-renewal, proliferation, differentiation and secreting various types of protection factors to sustain and improve cardiac contractile functions [1, 2]. Therefore, transplantation with BMSCs may be a promising regeneration strategy to repair myocardium infarction tissue. When BMSCs are exposed to ischemia or ischemic reperfusion (I/R) environment, the survival rates of donor cells are markedly reduced [3]. Emerging evidences showed that reactive oxygen species (ROS) are generated in the later stage of hypoxia and serum deprivation in vitro [4] and ischemia in vivo [5] and during the period of reperfusion of ischemic myocardial cells [6]. ROS induces cell apoptosis [7], thereby a major detrimental factor for therapeutic engrafted BMSC during myocardiac treatment. Therefore, it is of great interest to

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establish oxidative model and to find molecules with a capability of anti-apoptosis.

Lysophosphatidic acid (LPA) is an endogenous phospholipid signal factor, that is critical in regulating cell proliferation, migration, differentiation and apoptosis through six G protein-coupled LPA receptors, LPA_1–LPA_6 [8–11]. LPA receptors are coupled to at least three different G proteins, Gαi, Gαq, $G\alpha_{12/13}$ [12], that associate with various modulators regulating various downstream signal pathways. In our previous study, we showed that in patients with myocardial infarction, levels of serum LPA were significantly increased [13] and expressions of LPA receptors were also increased in rat myocardium post-acute myocardial infarction [13], suggesting the activation of LPA signaling after myocardial injury. We also described that LPA protects MSCs against hypoxia and serum deprivation-induced apoptosis [14, 15] In addition to BMSCs, other studies demonstrated that LPA inhibits apoptosis of intestinal epithelial cells, chronic lymphocytic leukemia cells, fibroblasts, Schwann cell, renal proximal tubular cells, H197 cells, human mesenchymal stromal cells and macrophages [16, 17]. Additionally, LPA was also shown to induce apoptosis of various types of cells including neurons, smooth muscle cells, myeloid progenitor TF-1 cells and epithelial cells [17]. Hence, the contradictory role of LPA on apoptosis prompted us to investigate whether LPA attenuates H_2O_2-induced apoptosis of BMSCs.

Autophagy is a major intracellular degradation and recycling pathway [18]. Autophagy is often activated by damaged protein and impaired organelles that are mostly generated from apoptotic cells [19, 20]. ROS not only induces cell apoptosis but also appears to be a major promoter of autophagy. In normal conditions, modest increase in autophagy promotes cell survival through generating amino acids and fatty acids from recycling the damaged macromolecules or organelles or long-lived proteins [21]. However, abnormal and excessive autophagy could cause cell death because of excessive self-digestion and degradation of important cellular constituents [22, 23]. However, whether autophagy plays a role in H_2O_2-induced cell apoptosis has not been studied.

In the present study, we examined the role of LPA in H_2O_2-induced BMSC apoptosis. We show that LPA can effectively attenuate H_2O_2-induced BMSC apoptosis, which is mainly through G_i-coupled LPA_3 receptor and this anti-apoptotic effect is independent of LPA-induced autophagy. Moreover we also show that H_2O_2-induced autophagy is beneficial to BMSC survival.

Materials and methods

Materials

Iscove's modified Dulbecco's medium (IMDM) and fetal bovine serum (FBS) were bought from Gibco (Grand Island, NY, USA). LPA (oleoyl C 18:1) and 3-(4-[4-([1-(2-chlorophenyl)ethoxy]carbonylamino)-3-methyl-5-isoxazolyl]benzylsulfanyl) propanoic acid (Kil6425) were from Avanti Polar Lipids (Alabaster, AL). The Annexin V-FITC Apoptosis Detection Kit was purchased from Oncogene (San Diego, USA). Hoechst 33342, anti-rat LC3B polyclonal antibody (catalog L7543) and autophagy inhibitor Bafilomycin A1 (Baf A1) were from Sigma (St Louis, MO, USA). Anti-rat caspase-3 antibody (catalog #9662), Becline 1 polymonol antibody (catalog #3495), horseradish peroxidase-conjugated secondary antibodies to rabbit or mouse, ERK1/2 inhibitor Uo126 and PI3 K/AKT inhibitor LY294002 were purchased from Cell Signaling Technology (CST) (Beverly, MA, USA). Anti-mouse SQSTM1/P62 monolcolonol antibody (catalog ab56416) was from the Abcam (Cambridge, UK). G_i protein inhibitor pertussis toxin (PTX) and autophagy promoter GF109203x (GFx) were obtained from ENZO Life Science (New York, USA). Lipofectamine^{TM} RiMAX, LPA_1-siRNA, LPA_3-siRNA, negative-siRNA and Opti-MEM were obtained from life Technologies (Ambion/Applied Biosystems Life Technologies, USA).

Cell culture

The BMSCs were isolated from three weeks old Sprague–Dawley rats as previously described [4]. All procedures in the present study were approved by the Animal Care Committee of National Centre for Cardiovascular Diseases and Fuwai Hospital. Briefly, bone marrow was harvested from tibia and femur of Sprague–Dawley rats (60–80 g, male) and seeded into cell culture flasks with IMDM containing 10 % FBS and 100 units/ml penicillin–streptomycin, then incubated at 37 °C in a humidified tissue culture incubator containing 5 % CO_2 and 95 % air. The medium was replaced with fresh media 24 h later and non-adherent cells were removed. After another 24 h, cells were washed two times with phosphate-buffered saline (PBS) and then changed to new IMDM complete medium. After 2 or 3 days, when the cells confluence reached about 80 %, these cells were digested with trypsin and sub-cultured in 1:3 ratios into new culture flasks. All cells used in this study were the passage 2 or 3.

Cell treatment

The BMSCs were seeded into 6-cm culture flasks or six orifice for 12 or 24 h. When the cell density reached 60–70 %, H_2O_2 at concentrations of 50, 100, 150, 200, or 250 μM were mixed in the IMDM without FBS for 4 h. Time-depended studies were then carried out at 0, 2, 4, 6, and 8 h post treatment in the concentration of 250 μM of H_2O_2. LPA at concentrations of 1, 5, 10, 25, 50 μM was separately pre-incubated in complete IMDM medium for 1 h. Inhibitors of $LPA_{1/3}$ receptors and G_i protein, Ki16425 (10 μM) and PTX (200 ng/ml), were pre-incubated with cells in complete medium for a predetermined time of 90 min and 16 h respectively. In another set of experiments, BMSC were treated with autophagy promoter GFx (10 μM) or inhibitor Baf A1 (10 μM) for 2 h.

Assessment of morphological changes

BMSCs were treated with 250 μM H_2O_2 in six orifice. Cell nuclear condensation and fragmentation were assessed using chromatin dye Hoechst 33342 as previously described [4]. Briefly, cells were fixed in 1 % glutaraldehyde for 30 min at room temperature, and washed with PBS twice, then stained by 5 μg/ml Hoechst 33342 for 10 min at room temperature followed by fluorescent microscopy. Apoptotic cells were identified by morphological alteration as fragmented and condensed apoptotic nuclei.

Flow cytometric analysis of cell apoptosis

Apoptosis cells were detected by Annexin V-FITC/PI Kit. Briefly, cells were collected and resuspended in 200 μL binding buffer containing 10 μL Annexin V for 15 min on ice avoiding light. Then 300 μL binding buffer containing 5 μL propidium iodide (PI) was mixed for 5 min and BMSCs were immediately analyzed by flow cytometric analysis (FACS). Approximately 10,000–20,000 cells were analyzed in each sample [24].

SiRNA knockdown of $LPA_{1/3}$ receptors and G_{i2}, G_{i3} proteins

Knockdown of LPA receptors and G_i proteins was carried out using small interfering RNA (siRNA) for indicated target gene in BMSCs using Lipofectamine™ RiMAX according to manufacture's instruction. LPA_1 Stealth siRNA duplexes (LPA_1-siRNA) targeting sequences: 5′-AUA AAU AGG GAA AUG GAA GCG GCG G-3′ and 5′-CCG CCG CUU CCA UUU CCC UAU UUA U-3′. LPA_3 Stealth siRNA duplexes (LPA_3-siRNA) targeting sequences: 5′-UAC ACC ACC ACC AUG AUG AAG AAG G-3′ and 5′-CCU UCU UCA UCA UGG UGG UGG UGU A-3′. G_{i2} Stealth siRNA duplexes targeting sequences: 5′-GAC ACC AAG GAG AUC UAC ACG CAC U-3′ and 5′-AGU GCG UGU AGA UCU CCU UGG UGU C-3′. G_{i3} Stealth siRNA duplexes targeting sequences: 5′-UCA GCU CAA UGA UUC UGC UUC AUA G-3′ and 5′-AUA UGA AGC AGA AUC AUU GAG CUG A-3′. The scrambled siRNA controls was used as a negative control.

Quantitative real time PCR (qRT-PCR)

Total RNA was isolated from BMSCs using trizol regent according to the manufacture's instruction. The isolated RNAs was quantified by NANODROP 2000 spectrophotometer. cDNA was generated from 2 μg total RNA using M-MLV reverse transcriptase and oligo (dT) 18 primer. qRT-PCR was performed using SYBR PCR master mix according to the manufacture's instructions in the Applied Biosystems 7300 (Foster city, CS, USA). All gene specific extron primers used in the present study were as follows: LPA_1: 5′-TCT TCT GGG CCA TTT TCA A-3′ and 5′-GCC GTT GGG GTT CTC GTT-3′; LPA_3: 5′-TGT CAA CCG CTG GCT TCT-3′ and 5′-CAG TCA TCA CCG TCT CAT TAG-3′. G_{i2}: 5′-AAGACCTGTCGGGCGTCATC-3′ and 5′-GCGCTCCAGGTCATTCAGGTA-3′; G_{i3}: 5′-TGAAGACTACAGGCATTGTGGAGAC-3′ and 5′-GTTCGGATCTTTGGCCACCTA-3′. The thermal profile for PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The results were normalized to internal parallel control of 18 s.

Protein extraction and western blot analysis

Cells were collected and rinsed with ice-cold PBS twice and then lysed in ice-cold lysis buffer for 30 min on ice. Cell lysates were centrifuged at 13,000×*g* for 5 min at 4 °C and the protein concentration was determined by the Bradford assay. Equal amounts of protein (20 μg/lane) were separated on 12 % SDS-PAGE gels by electrophoresis for western blotting analysis. The proteins were then transferred to nitrocellulose membranes using semi-dry electroblotting apparatus, and the membranes were blocked for 2 h at room temperature in 5 % skim milk. The membranes were incubated with primary antibody in 5 % BSA or skim milk over night at 4 °C. In the following day the members were washed three times and secondary antibody was added and incubated for 2 h. After washing, the members were processed for analysis using a Chemiluminescence Detection Kit (Pierce) as described by the manufacturer. The target signals were normalized to the β-actin signal and analyzed semi-quantitatively with Quantity One system.

Statistical analysis

Data was expressed as mean ± SD. Differences among groups were tested by one-way ANOVA. Comparisons between two groups were evaluated using Student's *t* test. Two-sided *P* values were used and $P < 0.05$ was considered statistically significant.

Results

H_2O_2-induced BMSC apoptosis is dose- and time-dependent

We previously reported that H_2O_2 induces BMSC apoptosis at various time points and successfully established an oxidative stress model [7]. It has been demonstrated that 0.12 mM H_2O_2 induces apoptosis of 21.85 ± 5.92 % cell population when cells were exposed to H_2O_2 for 24 h in IMDM containing 2 % FBS [7]. However, when cells are ischemic or in an ischemic/reperfusion microenvironment, serum deprivation and no reflow phenomenon result in nutrition deficiency [25, 26] and ROS production. Thus, we assessed the effect of H_2O_2 on BMSCs in serum-free medium. We treated BMSC cells with H_2O_2 at different concentrations, 50, 100, 150, 200, 250 μM for 4 h. We found that apoptotic phenotype became evident from 150 μM of H_2O_2 and reached maximum at 250 μM of H_2O_2. In normal cells, cell nucleus were equitable coloring circle or oval. In contrast, in apoptotic cells, nucleus became condensed or fragmented. As shown in Fig. 1a, Hoechst 33342 staining suggested that 50 and 100 μM H_2O_2 induced cell apoptosis approximately 10 % and apoptotic index was markedly increased to about 20–45 % when H_2O_2 concentration was at 150–250 μM. Additionally, when BMSC were exposed to 250 μM H_2O_2, cleavage of pro-caspase-3 became evident at 4-h and persists to 8 h post treatment (Fig. 1b). These data suggested that H_2O_2-induced rat MSC apoptosis dependently on its treated dose and time.

LPA inhibits BMSC apoptosis induced by H_2O_2

Our previous study indicated that LPA protects BMSCs from hypoxia and serum deprivation induced apoptosis [14], but whether LPA could inhibit H_2O_2-induced apoptosis is unclear. In this study, LPA was pre-treated for 1 h before exposure to H_2O_2 and persisted in the medium throughout the whole experiment process. Thus, we tested whether LPA display an anti-apoptotic role against H_2O_2-induced cell death. We thus exposed BMSCs to LPA at increasing concentrations (1, 5, 10, 25, 50 μM) and followed by treating these cells with 250 μM H_2O_2 for additional 4 h. As shown in Fig. 2a, control/normal cells display large regular nuclei with equitable coloring circle or oval. In contrast, cells treated with H_2O_2 appeared to have shrunken and fragmented nuclei. On the other hand, inclusion of LPA in H_2O_2-treatment markedly reduced H_2O_2-induced cell apoptosis, closely to the control levels (Fig. 2a). FACS analysis indicated that exposure of BMSCs to 250 μM H_2O_2 resulted in apoptosis in 35 % cells at early stage of apoptosis (Annexin V^+/PI^- cells) and 5 % cells at middle/late stage of apoptosis (Annexin V^+/PI^+ cells) (Fig. 2b). Inclusion of 10 μM LPA effectively protected cell from apoptosis, both at early and middle/late stages (Fig. 2b). Furthermore, compared to H_2O_2-treat group, cleavage of pro-caspase-3, a reliable marker of apoptosis was markedly decreased by LPA addition, (Fig. 2c). These data suggest that 10 μM LPA effectively attenuates rat BMSC apoptosis induced by 250 μM H_2O_2 treatment.

LPA inhibits H_2O_2-induced BMSC apoptosis through G_i-coupled LPA_3

Since both LPA_1 and LPA_3 are expressed in rat BMSCs (Fig. S1), we determined which LPA receptor mediates LPA protection. BMSCs were pre-treated with Ki16425 (10 μM), a specific antagonist of LPA_1 and LPA_3 receptors for 90 min or a G_i protein inhibitor PTX (200 ng/ml) for 16 h before exposure to H_2O_2 and LPA. As shown in Fig. 3a, Ki16425 and PTX significantly increase levels of cleaved-caspase-3 protein compared to LPA treatment only group (Fig. 3a, b, respectively). FACS analysis further revealed that Ki16425 and PTX could also markedly elevate the percentage of Annexin V^+/PI^- cells in the presence of LPA (Fig. 3c). Thus this data indicated that LPA primarily exhibits its anti-apoptosis effect against H_2O_2 through $LPA_{1/3}$ receptors coupling with G_i proteins.

To further determine which subtype of LPA receptors was involved in the anti-apoptosis effect of LPA, we separately knocked down LPA_1 and LPA_3 using specific siRNAs and test the apoptotic response of BMSCs induced by H_2O_2 with or without LPA treatment. As shown in Fig. 4a–c, LPA_1 and LPA_3 were both effectively knocked down in BMSCs (Fig. 4a, b) and inhibition of LPA_3 resulted in a greater increase in cleaved-caspase-3 proteins than that of LPA_1, indicating that LPA_3 mainly mediates the anti-apoptosis effect by LPA against H_2O_2 treatment. On the other hand, when G_{i2} or G_{i3} were knocked down by siRNAs (Fig. 4d), levels of cleaved-caspase-3 increased in BMSCs (Fig. 4e). These data suggested that LPA inhibits H_2O_2-induced apoptosis through G_i protein-coupled LPA_3 receptor.

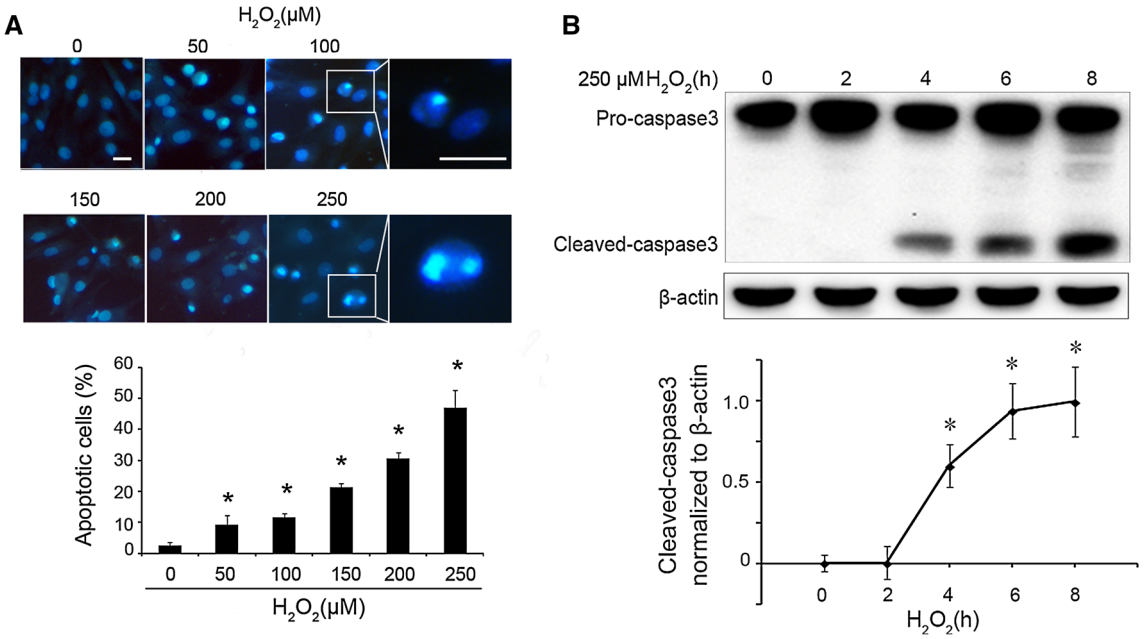

Fig. 1 H_2O_2 induces rat BMSC apoptosis in does- and time-dependent manner. **a** BMSCs were treated with H_2O_2 at indicated concentrations for 4 h and apoptotic degree was determined by Hoechst 33342 staining. *Scale bars* 10 μM. **b** BMSCs were exposed to 250 μM H_2O_2 for the pointed time and cell lysates were obtained and followed by western blot detection for cleaved-caspase-3 protein which was normalized to that of β-actin. All data are presented as mean ± SD of three independent experiments. $^*P<0.05$ when compared to control cells

ERK1/2- and PI3 K/AKT-signaling pathways are involved in LPA-mediated anti-apoptosis effect in BMSCs

It is established that LPA exerts its anti-apoptotic function primarily through ERK1/2- and/or PI3 K/AKT-signaling pathway [14]. Thus we determined whether activation of ERK1/2 and PI3 K signaling is involved in LPA-LPA_3 protection against H_2O_2-induced BMSC apoptosis. As shown in Fig. 5a, b, after exposure of BMSC to LPA for 5 or 10 min, phosphorylation of ERK1/2 and AKT were significantly elevated and the LPA induction of p-ERK1/2 and p-AKT was markedly attenuated by inclusion of Ki16425 or PTX (Fig. 5c, d). As expected, both ERK inhibitor U0126 and PI3 K inhibitor LY294002 blocked LPA's anti-apoptosis protection as indicated by cleaved-caspase-3 expression (Fig. 5e) and the early stage of apoptosis (Annexin V^+/PI^-) (Fig. 5f). These data indicate that ERK1/2 and PI3 K/AKT pathways are involved in LPA protection of BMSC from H_2O_2-induced apoptosis.

Autophagy induced by LPA has no connection with its anti-apoptotic effect against H_2O_2

Oxidative stress was shown to induce autophagy [27] which had dual functions for cell survival [23, 28]. Thus, we investigated effects of H_2O_2 and LPA on the autophagy of rat BMSCs and its relation to cell apoptosis by examining status of LC3BII/I, BECN and P62 that are common biochemical makers of autophagy, and treating BMSCs with GF109203x, an autophagy promoter, and Baf A1, an autophagy flux inhibitor. Then we determined whether the induced autophagy is beneficial to cell survival of H_2O_2-treated BMSCs. As shown in Fig. 6a, treatment of BMSC with 250 μM H_2O_2-induced a time-dependent autophagy indicated by the increase of LC3BII/I ratio and the decrease of P62 expression. Inclusion of 10 μM LPA further enhanced the conversion of LC3BI to LC3BII without further decrease in P62 expression in the presence of 250 μM H_2O_2, (Fig. 6b), suggesting that LPA further promotes H_2O_2-induced autophagy which may be involved in formation of autophagsome related to LC3II/I and is not linked to P62-related autophagy flux. Based on these observations, we next, tested whether the 10 μM LPA promotion of autophagy contribute to its anti-apoptosis effect. As shown in Fig. 6c, the autophagy promoter GFxsignificantly decreased H_2O_2-induced elevation of LC3II/I ratio and levels of cleaved-caspase-3, respectively (Fig. 6c). And Baf A1, the autophagy flux inhibitor failed to improve the cell survival (Fig. 6c). These results further indicate that strengthening autophagy by H_2O_2 may benefit for cell survival. However, as shown in Fig. 6d, LPA significant inhibited H_2O_2-caused cleavage of pro-caspase-3, but the inhibition did not been changed by the inclusion of

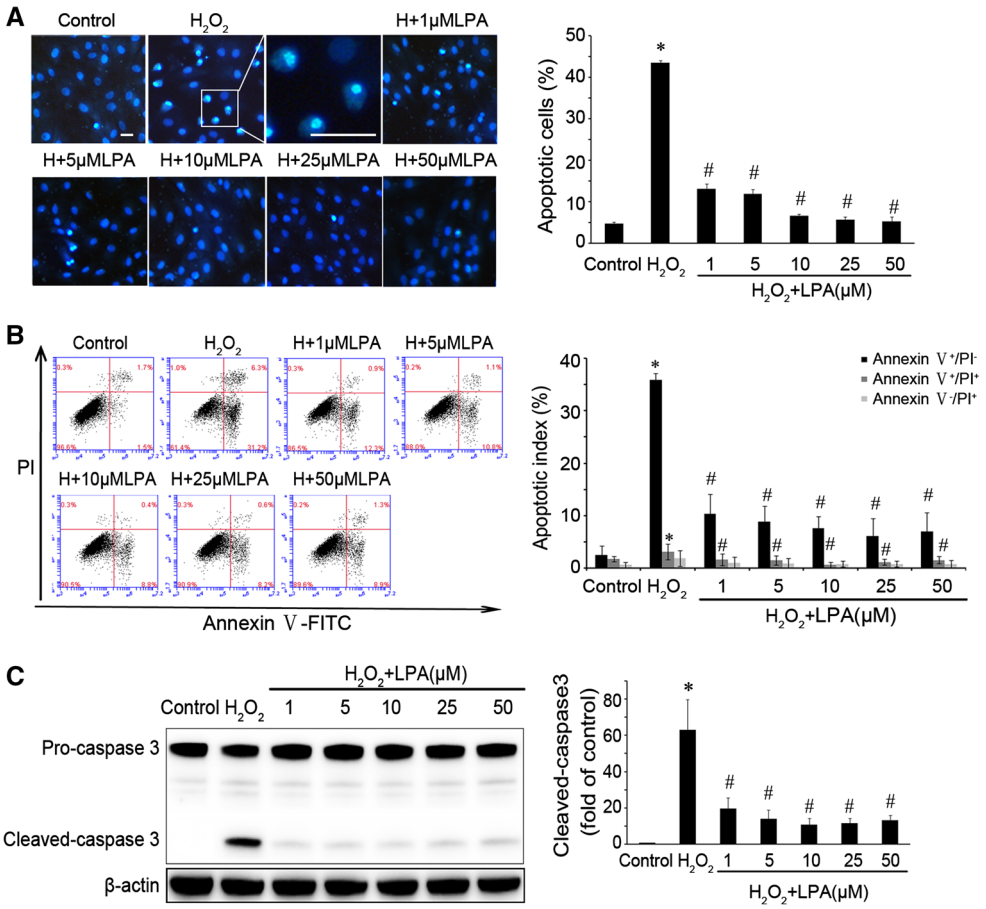

Fig. 2 LPA inhibits H_2O_2-induced apoptosis in BMSCs. BMSCs were pre-treated with LPA at indicated concentration for 60 min in complete IMDM medium before exposure to H_2O_2 (250 μM) for 4 h. Cell apoptosis was determined by Hoechst 33342 staining (**a**), FACS analysis of apoptotic cells with AnnexinV-FITC and propidine iodide (PI) staining: Annexin V^+/PI^- cells represents early apoptotic cells; Annexin V^+/PI^+ represents late apoptotic cells; Annexin V^-/PI^+ represents necrotic cells (**b**) and western blot analysis of cleaved-caspase-3 protein (**c**). "H" represents of "H_2O_2". *Scale bars* 10 μm. *Bar graphs*, quantitation of indicated results. Each column represents the mean ± SD of three independent experiments. $^*P<0.05$ when compared to the control cells. $^\#P<0.05$ when compared to the H_2O_2-treated cells

Baf A1, indicating that LPA rescue of BMSCs from H_2O_2-induced apoptosis was independent of autophagy.

Discussion

In our study, we characterized H_2O_2-induced BMSC apoptosis under the condition of serum-free/nutrition deficiency. Our data showed that LPA could effectively inhibit H_2O_2-induced BMSC apoptosis mainly by binding to G_i protein-coupled LPA_3 receptor that in turn activates ERK1/2- and PI3 K/AKT-signaling pathways. We also found that H_2O_2 induces autophagy in BMSC that is beneficial to cell survival. However, LPA enhanced H_2O_2-induced autophagy in BMSCs is independent of its anti-apoptosis action against H_2O_2.

BMSCs are showing great therapeutic potential for ischemic heart disease and heart failure. When myocardial infarction occurs, hypoxia and hypoxia-inducible factor-1α in ischemic injury lead to ROS bursting such as O_2^- and H_2O_2 within mitochondria under NADPH oxidase catalyst [29]. O_2^- produced from oxygen can be quickly converted to H_2O_2 by superoxide dismutase (SOD) [30]. Additionally, ischemia/reperfusion (I/R) can bring about abundant of oxygen radical, for example OH^- produced from H_2O_2. Low level of H_2O_2 maintains HSC stemness whereas high level of H_2O_2 stimulates cell proliferation, differentiation, migration and reduces cell survival [31]. H_2O_2, as one of the most stable ROS, was often used to establish oxidative stress injury model in vitro for the apoptosis and anti-apoptosis mechanism research [32]. At the present study, dose- and time-experiments revealed that 250 μM of H_2O_2 strongly induced apoptosis of BMSCs at 4 h post-treatment, suggesting the successfully building of acute oxidative stress injury model (Fig. 1). This data indicates that exogenous ROS burst in the microenvironment of myocardial ischemic and reperfusion maybe a critical inducement of BMSCs' apoptosis. These results also warrant a necessity to develop new molecules and drugs to rescue BMSCs from ROS-induced apoptosis.

Recently, studies of anti-apoptosis role against H_2O_2 have drawn attention of in cardiovascular research field. Our previous study described that LPA, a pleiotropic lipid growth factor effectively inhibits apoptosis of BMSCs against hypoxia and serum starvation [14]. Other investigations also reported that LPA is capable of preventing cell apoptosis of human dental pulp cells [33], intestinal

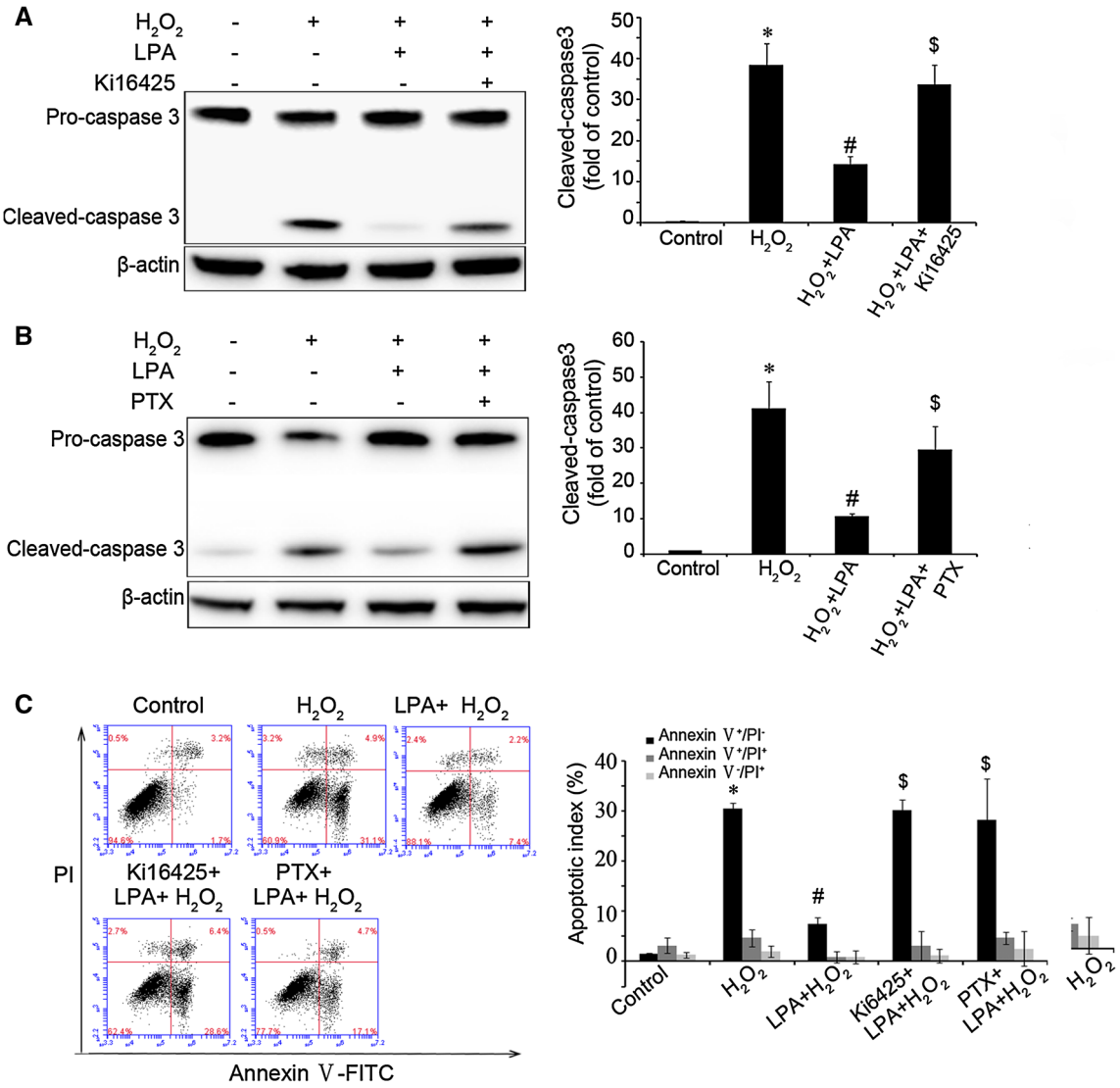

Fig. 3 LPA protects BMSCs from H_2O_2-induced apoptosis through G_i-coupled $LPA_{1/3}$. Cell apoptosis was detected by western blot analysis for expression of cleaved-caspase-3 (**a** and **b**) and FACS analysis after staining with AnnexinV and PI (**c**). BMSCs that were pre-treated with 10 μM Ki16425 for 90 min or 200 ng/ml PTX for 16 h before exposure to 10 μM LPA for 60 min followed by 250 μM H_2O_2 for an additional 4 h. The results are presented as mean ± SD and representative of three independent experiments. *Bar graphs*, quantitation of indicated results. $^{*}P<0.05$ when compared to the control cells. $^{\#}P<0.05$ when compared to the H_2O_2 group. $^{\$}P<0.05$ when compared to LPA + H_2O_2 group

epithelial cells [34], H19-7 cells (an embryonic hippocampal progenitor cell line) [35] and human mesenchymal stromal cells [36]. Hence, whether LPA could play its anti-apoptosis role against H_2O_2 is of high interest. This study, for the first time, tested and validated this hypothesis and established a protective role of LPA against H_2O_2-iduced apoptosis of rat BMSCs. This research lay an important basis for applying potential of LPA in therapeutic strategy of myocardial infarction through stem cell transplantation.

LPA mediates a wide range of cellular function through different LPA receptor subtypes that coupled to specific G protein. Determining the exact role of each receptor subtype will provide promising targets for therapeutic intervention. The expression profiles of LPA receptors are often different from different cell types. We previously reported that LPA_1 mediates LPA-induced apoptosis while LPA_3 is responsible for the pro-proliferation role in neonatal rat cardiac fibroblasts [37]. A recent study also revealed that LPA_1 and LPA_3 play opposite roles on cell motile and invasive activities in pancreatic cancer cells [38]. Hence, distinguishing the subtypes of LPA receptors and their specific function will contribute to develop proper and effective therapeutic target. In this study, LPA_3 displayed greater protective role against H_2O_2 than LPA_1 in

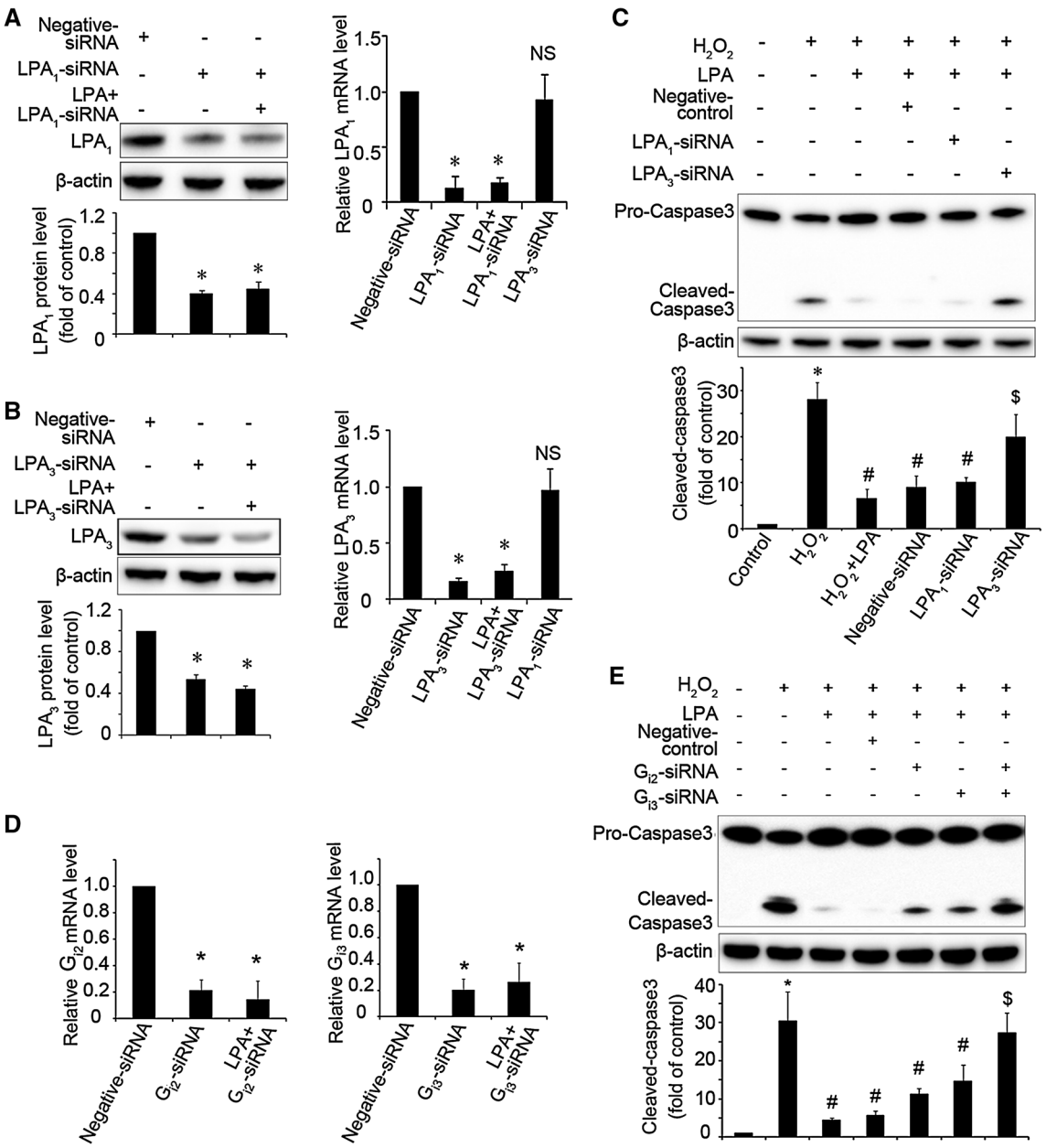

Fig. 4 LPA_3, G_{i2} and G_{i3} mediate the anti-apoptosis of LPA against H_2O_2 in rat BMSCs. BMSCs were separately transfected with control siRNAs or siRNAs specific for LPA_1, LPA_3 (**a**–**c**), G_{i2},or G_{i3} (**d** and **e**) for 24 h followed by H_2O_2 (250 μM) treatment for an additional 4 h. mRNA levels of LPA_1 and LPA_3 were determined by qRT-PCR. Protein levels of G_{i2}, G_{i3}, LPA_1, LPA_3, and caspase-3 were assessed by western blot. *Bar graphs*, quantitation of indicated results. The results are presented as mean ± SD and representative of three independent experiments. $^{*}P<0.05$ when compared to the control cells. $^{\#}P<0.05$ when compared to the H_2O_2 group. $^{\$}P<0.05$ when compared to LPA + H_2O_2 group. $^{\triangle}P<0.05$ when compared to the control group. *NS* no difference

the presence of LPA. In addition, G_i proteins were shown to regulate different biological processes including the pro-proliferation of cervical cancer cells by coupling to different receptors of LPA [39], regulation transformation of mouse embryo fibroblasts [40] and the pro-migration of rat hepatoma RH7777 cell [41]. Our data corroborates with these studies. We show that both G_{i2} and G_{i3} proteins are involved in LPA-LPA_3 protection of H_2O_2-induced cell apoptosis in BMSC. Taken together, our data suggests that LPA play its anti-apoptosis effect mainly through G_i protein coupled LPA_3 receptor. Thus, G_i-LPA_3 might be an important therapeutic target for BMSC survival.

It is established that LPA signaling through PTX-sensitive G_i protein activates ERK1/2 and PI3 K/AKT pathways most commonly contributes to the LPA-elicited anti-apoptotic action [42]. In our study, we found that LPA

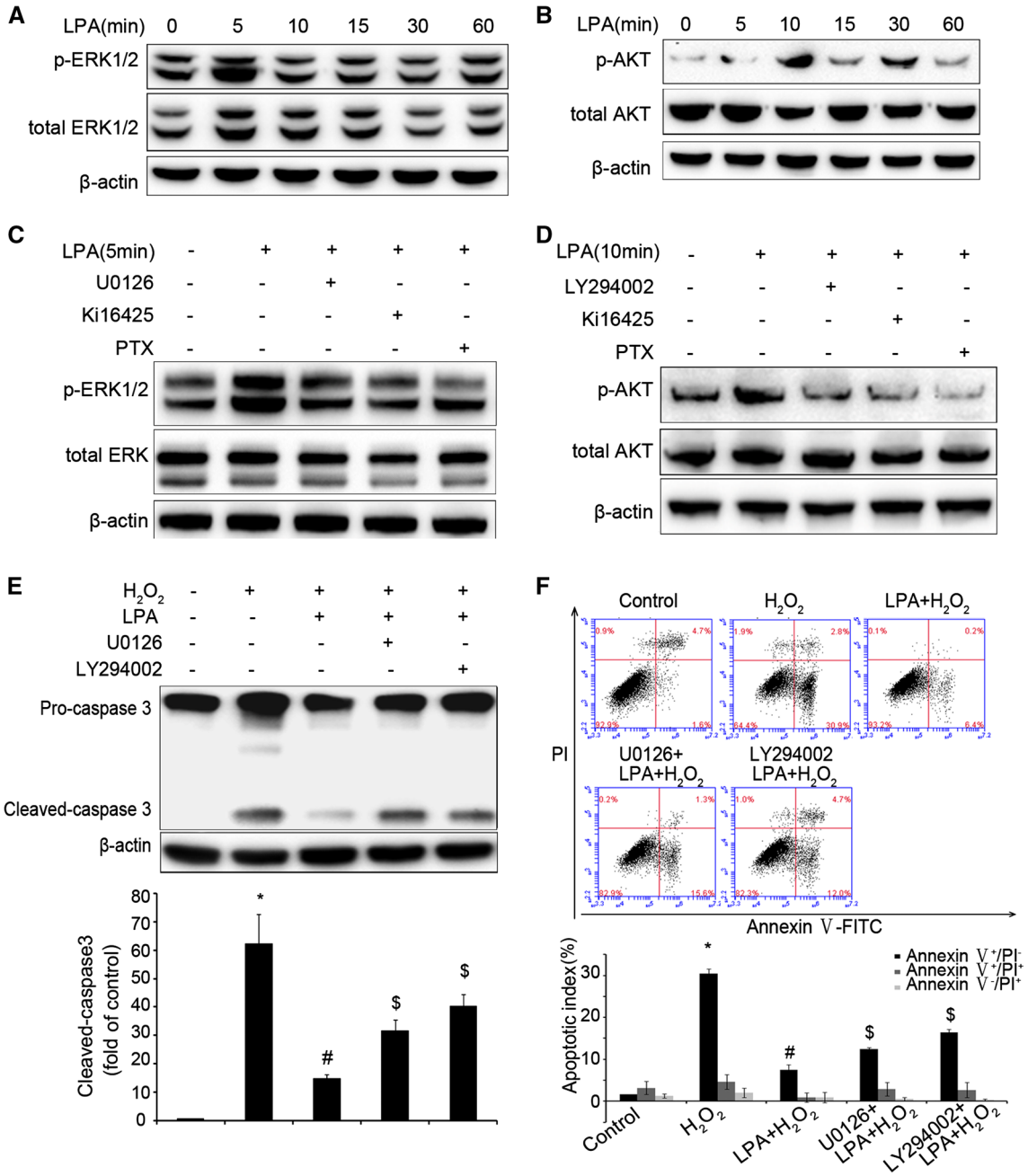

Fig. 5 ERK1/2 and PI3 K/AKT pathways are involved in the anti-apoptotic effect of LPA against H_2O_2 in rat BMSCs. Western blot were used for testing p-ERK1/2 and p-AKT in BMSCs that were exposed to 10 μM LPA for the indicated time (**a** and **b**), 5 min (**c**), or 10 min (**d**) after 10 μM Ki16425 and 200 ng/ml PTX treatment for 90 min and 16 h respectively. BMSCs were pre-treated with ERK1/2 inhibitor U0126 (10 μM) or PI3 K/AKT inhibitor LY294002 (25 μM) for 90 min before their exposure to LPA (10 μM) and H_2O_2 (250 μM) for 4 h. Cleaved-caspase-3 was determined by western blot (**e**) and apoptotic cells were assessed by FACS analysis following Annexin V and PI staining (**f**). *Bar graphs*, quantitation of indicated results. Each data point (±SD) is representative of three independent experiments. $^{*}P<0.05$ when compared to control cells. $^{\#}P<0.05$ and $^{\$}P<0.05$ when compared with the LPA + H_2O_2 group

markedly induces p-ERK1/2 and p-AKT in rat BMSCs that are inhibited after pretreatment of Ki16425 and PTX. Blocking these two pathways separately could partly prevent LPA-elicited anti-apoptotic activity, indicating that ERK1/2 and PI3 K/AKT simultaneously took part in the anti-apoptotic process by LPA. However, the downstream signal mediating ERK1/2 and PI3 K/AKT converge and downstream target need to be identified.

Autophagy, as a scavenger inside our body, often occurs at basal condition and mediate homeostatic balance [43]. Increasing lines of evidence suggest that autophagy is activated during many pathologic conditions in heart such

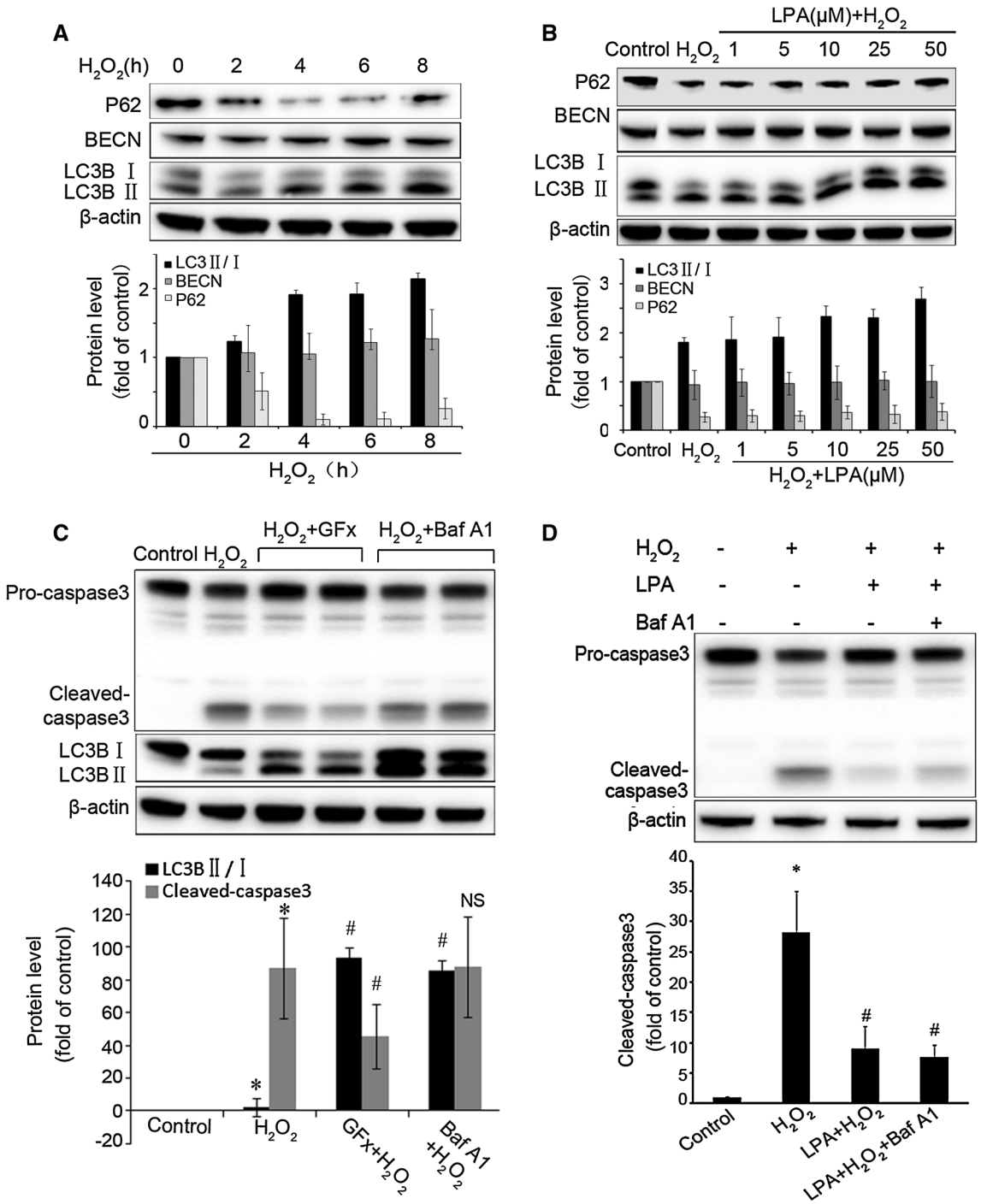

Fig. 6 LPA-induced autophagy is independent of the anti-apoptotic effect of LPA against H_2O_2. Rat BMSCs were treated with 250 μM H_2O_2 at indicated times (**a**) or for 4 h after incubation with LPA at indicated concentrations for 1 h (**b**) or for 4 h following the exposure to an autophagy promoter GF109203x (GFx) or an authophagy inhibitor Bafilomycin A1 (Baf A1) for 90 min (**c**) followed by incubation with LPA for an additional 1 h (**d**). Western blot was used to detect LC3B II/I ratio, BECN, P62 and cleaved-caspase-3 expression. The two plots (H_2O_2 + GFx) and (H_2O_2 + Baf A1) each in (**c**) are replicates. β-actin was used as loading control. Bar graphs, quantitation of indicated results. Each column represents the mean ± SD of three independent experiments. $^{*}P<0.05$ and $^{\#}P<0.05$ when compared to H_2O_2-treated only group. *NS* no difference

as chronic and acute ischemia in the myocardium [44], I/R [45], cardiac hypertrophy [46] and heart failure [47]. These diseases bring about environment stresses such as nutrient starvation, hypoxia, endoplasmic reticulum (ER) stress and oxidative stress which are the major causes of autophagy activation [48]. Autophagy is known to play a protective

effect in ischemic myocardial but detrimental role in I/R's different processing stages [27, 44]. ROS, a major product of hypoxia and oxidative stress can promote autophagy which can protect themselves from stress injury [49] or further promote cellular senescence [50]. In this study, we found that H_2O_2 induces autophagy in time- and dose-dependent manner. Autophagy agonist GFxcould partly inhibit BMSC apoptosis, indicating H_2O_2 induced autophagy is beneficial to cell survival in BMSCs.

Although LPA has been proved to affect cell apoptosis, including our present study, little is known about the influence of LPA over autophagy. In this study, we found that LPA promotes autophagy of BMSCs in the presence of H_2O_2, despite the autophagy did not contribute to its anti-apoptotic action. Indeed, (S1P), a simple lysophospholipid molecule similar to LPA, can induce autophagy to protect human prostate cancer PC-3 cells and Human breast cancer MCF-7 cells from apoptosis [51, 52] while ceramide induced autophagic cell death in malignant glioma cells via activation of BNIP3 [53]. Therefore, this study as well as other reports provides new directions for the in-depth study of LPA-induced autophagy.

In summary, we demonstrate that H_2O_2 induces BMSCs apoptosis in time- and dose-dependent manner. LPA inhibits H_2O_2-induced apoptosis mainly by binding to G_i-coupled LPA_3 receptor to activate ERK1/2 and PI3 K/AKT signaling pathways. LPA can promote H_2O_2-induced autophagy which has no connection with the anti-apoptosis role of LPA. Our findings reveal an important anti-apoptosis function of LPA against H_2O_2, which may provide a potential and effective therapeutic strategy for cardiac regeneration and heart repair.

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Conflict of interest The authors declare that they have no conflict of interest.

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