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LPA Rescues ER Stress-Associated Apoptosis in Hypoxia and Serum Deprivation-Stimulated Mesenchymal Stem Cells

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ABSTRACT

Poor viability of transplanted mesenchymal stem cells (MSCs) in the infracted heart has limited their therapeutic efficacy in cardiac repair after myocardial infarction. We previously demonstrated that hypoxia and serum deprivation (hypoxia/SD) induced mitochondria-dependent apoptosis in MSCs, while lysophosphatidic acid (LPA) could almost completely block this apoptotic process. However, the role of endoplasmic reticulum (ER) stress and its upstream signaling events in hypoxia/SD-induced MSC apoptosis remain largely unknown. Here we found that hypoxia/SD-induced MSC apoptosis was associated with ER stress, as shown by the induction of CHOP expression and procaspase-12 cleavage, while the effects were abrogated by LPA treatment, suggesting ER stress is also a target of LPA. Furthermore, hypoxia/SD induced p38 activation, inhibition of which resulted in decreases of apoptotic cells, procaspase-12 cleavage and mitochondrial cytochrome *c* release that function in parallel in MSC apoptosis. Unexpectedly, p38 inhibition enhanced hypoxia/SD-induced CHOP expression. Interestingly, p38 activation, a common process mediating various biological effects of LPA, was inhibited by LPA in this study, and the regulation of p38 pathway by LPA was dependent on $LPA_{1/3}$/Gi/ERK1/2 pathway-mediated MKP-1 induction but independent of PI3K/Akt pathway. Collectively, our findings indicate that ER stress is a target of LPA to antagonize hypoxia/SD-induced MSC apoptosis, and the modulation of mitochondrial and ER stress-associated apoptotic pathways by LPA is at least partly dependent on $LPA_{1/3}$/Gi/ERK/MKP-1 pathway-mediated p38 inhibition. This study may provide new anti-apoptotic targets for elevating the viability of MSCs for therapeutic potential of cardiac repair. J. Cell. Biochem. 111: 811–820, 2010.

KEY WORDS: LYSOPHOSPHATIDIC ACID; P38 MAPK; MESENCHYMAL STEM CELL; HYPOXIA/SERUM DEPRIVATION; ENDOPLASMIC RETICULUM STRESS; APOPTOSIS

Mesenchymal stem cells (MSCs) are a unique subset of stem cells that can be isolated from the bone marrow, adipose tissue, and even umbilical cord blood [Kestendjieva et al., 2008; Kumar et al., 2008]. In response to ischemia-reperfusion injury, MSCs secrete a variety of cytokines that are cardioprotective or angiogenic [Kinnaird et al., 2004; Amado et al., 2005]. In addition, MSCs can be engrafted into injured myocardium and potentially differentiate into cardiac myocytes [Amado et al., 2005; Miyahara et al., 2006]. However, the efficacy of MSCs transplantation strategy is limited by the poor viability of MSCs transplanted into the infracted heart [Mangi et al., 2003; Hanabusa et al., 2005; Tang et al., 2005]. Therefore, it is essential to have a clear understanding of the events and factors that may predispose MSCs to undergo apoptosis in ischemic tissue.

Accumulating evidence shows that the endoplasmic reticulum (ER) plays a vital role in integration of proapoptotic signaling [Ferri and Kroemer, 2001]. Disturbances in the normal functions of ER lead to the evolutionarily conserved unfolded protein response (UPR), which is mediated through three ER transmembrane receptors: PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) [Xu et al., 2005]. In resting cells, all of three receptors are maintained in an inactive state through association with GRP78, an ER resident chaperone [Fu et al., 2008], which is also reported to be expressed on some tumor cell surfaces,

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functioning as a receptor for a wide variety of ligands [Gonzalez-Gronow et al., 2007]. Upon ER stresses, GRP78 dissociates from the ER transmembrane receptors, which will lead to their activation and trigger UPR. Activation of IRE1 induces X box-binding protein (XBP-1) mRNA splicing [Lee et al., 2002]. This spliced form of XBP-1 mRNA encodes an active transcription factor which induces expression of a subset of genes encoding chaperones as well as genes encoding protein degradation enzymes [Lee et al., 2003]. Activation of PERK leads to phosphorylation of the eukaryotic initiation factor eIF2α [Harding et al., 2000], thereby suppressing most of the de novo protein synthesis during ER stress [Krishnamoorthy et al., 2001]. The UPR is a prosurvival response to reduce the accumulation of unfolded proteins and restore normal ER function [Szegezdi et al., 2006]. If these adaptive responses are insufficient to protect the cells from ER stress, then ER will serve as a site where apoptotic signals are generated through several pathways, including induction of the transcription factor CHOP (also known as GADD153) [Oyadomari and Mori, 2004], proteolysis-induced activation of procaspase-12 [Nakagawa et al., 2000] and activation of the c-Jun N-terminal kinase (JNK) cascade through the ER membrane protein IRE1 [Urano et al., 2000].

Mitogen-activated protein kinases (MAPKs) consist of three family members: the extracellular signal-regulated kinases (ERK), JNK, and p38 MAPK [Raman et al., 2007]. ERK is generally considered to be a survival factor involved in the protective actions of growth factors in apoptosis [Walker et al., 1998; Poynter et al., 1999], whereas p38 and JNK are usually required for induction of apoptosis by diverse stimuli [Nagai et al., 2007]. However, the individual roles of JNK and p38 remain to be clarified in different cell types and conditions. Since the complex phosphorylation-dependent signaling pathways depend on the balance between kinase and phosphatase activity, several studies have turned to identify specific phosphatases for each kinase in the cascade. MAPK inactivation is performed by a family of MAPK phosphatases (MKPs) [Keyse, 2000]. The archetypal member of MKPs, MAPK phosphatase 1 (MKP-1), selectively inactivates all three MAPK families by dephosphorylating them at catalytic tyrosine and threonine residues, but it acts preferentially on p38 and JNK [Franklin and Kraft, 1997].

Lysophosphatidic acid (LPA, 1-acyl-2-hydroxy-sn-glycero-3-phosphate) is a naturally occurring bioactive lipid with multiple functions in biological systems, such as oncogenesis, wound healing, cell proliferation, migration and survival [van Meeteren and Moolenaar, 2007]. The multitude of activities of LPA is consistent with the broad tissue distribution of LPA receptors, which are coupled to at least three different heterotrimeric G proteins: Gαi, Gαq, $G\alpha_{12/13}$ [Anliker and Chun, 2004]. Briefly, the major G protein-linked effector pathways include: (i) Gαq-mediated stimulation of phospholipase C (PLC); (ii) $G\alpha_{12/13}$-mediated activation of the small GTPase RhoA; and (iii) Gαi-mediated inhibition of adenylate cyclase with a subsequent fall in cAMP levels; stimulation of the Ras-MAP kinase cascade; activation of phosphoinositide-3-kinase (PI3K) with subsequent activation of Rac and PKB/Akt [Moolenaar et al., 2004].

We previously demonstrated that in response to hypoxia and serum deprivation (hypoxia/SD), both of which are components of ischemia in vivo, MSCs underwent the mitochondria-dependent apoptosis [Zhu et al., 2006], while LPA could almost completely block hypoxia/SD-induced apoptosis in MSCs by activating ERK1/2 and PI3K/Akt pathways [Chen et al., 2008]. However, whether there are other proapoptotic pathways existing that mediate hypoxia/SD-induced MSC apoptosis remains largely unknown. Herein, we focused on investigating the roles of ER stress-associated apoptotic pathways and the effects of LPA on these pathways in hypoxia/SD-induced MSC apoptosis. Furthermore, our studies were extended to identify the signaling mechanisms that mediated such actions of LPA.

MATERIALS AND METHODS

MATERIALS

Iscove's modified Dulbecco's medium (IMDM) and fetal bovine serum were from Gibco (Grand Island, NY). Trizol reagent was purchased from Invitrogen (Carlsbad, CA). M-MLV reverse transcriptase was from Promega (Madison, WI). LPA (oleoyl C: 18:1) and DGPP (8:0) were from Avanti Polar Lipids (Alabaster, AL). Pertussis toxin (PTX) and wortmannin were from Biomol Research Labs (Plymouth Meeting, PA). Power SYBR green PCR master mix was from Applied Biosystems (Foster City, CA). Mitochondrial membrane potential assay kit with JC-1 and cell mitochondria isolation kit were provided by Beyotime Institute of Biotechnology (Haimen, China). Annexin V-FITC apoptosis detection kit was purchased from Oncogene (San Diego, CA). Z-ATAD-FMK was from Biovision (Mountain View, CA). Hoechst 33342, SB202190, Ki16425, SP600125, U0126 and the antibody for caspase-12 were obtained from Sigma (St. Louis, MO). Antibodies for p-eIF2α and eIF2α were purchased from Bioworld Technology (Minneapolis, MN). Antibodies for phospho-Akt, p38, and caspase-3 were obtained from Cell Signaling Technology (Danvers, MA). Antibodies for phospho-p38, cytochrome *c*, CHOP, XBP-1, Akt, β-actin and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

CELL CULTURE AND TREATMENT

Bone marrow was harvested from the tibia and femur of 80-g rats, plated in IMDM supplemented with 15% inactivated fetal bovine serum and 100 units/ml penicillin/streptomycin and incubated at 37°C in a humidified tissue culture incubator containing 5% CO_2 and 95% air. The medium was replaced 4 h after plating and 24 h later to discard nonadherent hematopoietic cells. Adherent MSCs were further grown in medium replaced every 2 days [Chen et al., 2008]. All procedures in the present study were approved by the Animal Care Committee of Cardiovascular Institute and Fuwai Hospital. The cells used in the present study were from passages 1 to 3. Cells exposed to hypoxia/SD alone were used as the apoptotic controls and this was induced by incubating MSCs in serum-free media in a sealed, hypoxic GENbox jar fitted with a catalyst (BioMe'rieux, Marcy l'Etoile, France) to scavenge free oxygen. Oxygen tension in the medium was measured using a blood gas analyzer and was found to be 33.5 mm Hg within 0.5 h after being transferred into the hypoxic chamber and maintained at approximately 22–24 mm Hg over the experimental time. Cells cultured in complete medium alone were used as the nonischemic controls.

When used, 15 μM SB202190 (p38 inhibitor) or 2.5 μM Z-ATAD-FMK (caspase-12 inhibitor) were preincubated with cells in complete medium for 1 h. Cells were subsequently washed in serum-free IMDM and exposed to hypoxia/SD for 6 h in the continued presence of each inhibitor. While 50 μM DGPP or 10 μM Ki16425 (LPA_1/LPA_3 antagonists), 100 nM wortmannin (PI3K inhibitor) or 20 μM U0126 (ERK inhibitor) were preincubated with cells for 80 min, and then LPA (10 μM) was added in the presence of each drug for 1 h. Cells were subsequently exposed to hypoxia/SD in the continued presence of LPA and a selected inhibitor. In experiments involving Gi proteins, MSCs were preincubated with 200 ng/ml PTX for 16 h. LPA (10 μM) was added for the last hour of preincubation when required. Cells were then exposed to hypoxia/SD in the continued presence of PTX and LPA.

RNA EXTRACTION AND REAL-TIME PCR ANALYSIS

Total RNA was extracted from MSCs using Trizol reagent according to the manufacturer's instructions and cDNA was generated from 2 μg of total RNA using M-MLV reverse transcriptase and oligo(dT)18 primer. Real-time PCR was performed in total volume of 25 μl containing 0.5 μl RT product, 0.5 μM primers, and 12.5 μl SYBR Green PCR Master mix. GAPDH mRNA amplified from the same samples served as an internal control. The relative expression levels of each targeted gene were normalized by subtracting the corresponding GAPDH threshold cycle (Ct) values by using the ΔΔCt comparative method.

The sequences of all primers used in this work are depicted as follows: GRP78: 5′-GCTGGCACTATTGCTGGACTGA-3′ and 5′-AGACACATCGAAGGTTCCACCAC-3′; Caspase-12: CAATTCCGACAAACAGCTGAGTTTA and CATGGGCCACTCCAACATTTAC; CHOP: 5′-CAGCGACAGAGCCAAAATAACA-3′ and 5′-CAAAGGCGAAAGGCAGAGA-3′; MKP-1: 5′-ACAACCACAAGGCAGACATTAGCTC-3′ and 5′-CAGATGGTGGCTGACCTGGA-3′; GAPDH: 5′-GGCACAGTCAAGGCTGAGAATG-3′ and 5′-ATGGTGGTGAAGACGCCAGTA-3′.

MORPHOLOGICAL EXAMINATION OF APOPTOTIC CELLS

Chromosomal condensation was assessed using the chromatin dye Hoechst 33342. Cells were fixed for 30 min in phosphate-buffered saline (PBS) containing 1% glutaraldehyde. After fixing at room temperature, the cells were washed twice with PBS and then exposed to 5 μg/ml of Hoechst 33342 in PBS for 30 min at room temperature. All samples were observed using a fluorescence microscope. Apoptotic cells were characterized by morphological alteration as condensed nuclei and cell shrinkage.

FLOW CYTOMETRIC ANALYSIS OF CELL APOPTOSIS AND MITOCHONDRIAL MEMBRANE POTENTIAL

Apoptosis was determined by detecting phosphatidylserine (PS) exposure on cell plasma membrane with the fluorescent dye Annexin V-FITC apoptosis detection kit as previously described [Chen et al., 2008], which can discriminate intact (Annexin V^-/PI^-), early apoptotic (Annexin V^+/PI^-), late apoptotic (Annexin V^+/PI^+), and necrotic cells (Annexin V^-/PI^+).

JC-1 was used to demonstrate the change in the mitochondrial membrane potential ($\Delta\Psi m$) of MSCs. Briefly, cells were harvested after indicated treatments and resuspended in 500 μl of complete medium with an equal volume of JC-1 staining solution (5 μg/ml) at 37°C for 20 min in the dark. After washing with JC-1 wash buffer, cells were resuspended in 1 ml JC-1 wash buffer, and immediately analyzed by flow cytometry at 488 nm excitation. Results were presented in relative aggregate to monomer (red/green) fluorescence intensity ratio.

PROTEIN EXTRACTION, MITOCHONDRIAL ISOLATION, AND WESTERN BLOTTING ANALYSIS

Preparation of total cell lysates from stimulated cells for Western blot analysis was carried out as previously described [Chen et al., 2008]. For the analysis of mitochondrial cytochrome *c* release, 5×10^7 cells were harvested and washed with ice-cold PBS. Cells were incubated with 1.0 ml of 1× cytosol extraction buffer mix provided in the kit for 15 min, and then homogenized using an ice-cold Dounce Tissue Grinders. The homogenates were centrifuged at 600*g* for 10 min and then the supernatants further centrifuged at 11,000*g* for 10 min at 4°C. The cytosolic supernatants were decanted and the pellets resuspended in 0.1 ml mitochondrial extraction buffer mix. Cytochrome *c* released into the cytosol from mitochondria and that in mitochondria were then, respectively, determined by Western blotting using the cytochrome *c* antibody.

STATISTICAL ANALYSIS

Data are expressed as mean ± SEM. Differences among groups were tested by one-way ANOVA. Comparisons between two groups were evaluated using Student's *t*-test. A value of $P < 0.05$ was considered as significantly different.

RESULTS

INHIBITION OF p38 PROTECTS MSCs FROM HYPOXIA/SD-INDUCED APOPTOSIS

We have previously demonstrated that in response to hypoxia/SD, MSCs underwent the caspase-dependent apoptosis involving changes in mitochondrial integrity and function [Zhu et al., 2006]. Since there is evidence that p38 acted at the early step prior to mitochondrial dysfunction [Ghatan et al., 2000; Kim et al., 2006; Gomez-Lazaro et al., 2007], the kinetic p38 phosphorylation levels induced by hypoxia/SD were investigated. As shown in Figure 1A, rapid phosphorylation of p38 occurred after hypoxia/SD treatment. Next, to determine the contribution of p38 activation to hypoxia/SD-induced apoptosis of MSCs, the cells were exposed to 15 μM SB202190, a p38 specific inhibitor, followed by exposure to hypoxia/SD for 6 h. Cell death was analyzed morphologically and by flow cytometry. As shown in Figure 1B, control cells had the normal nuclei, but hypoxia/SD-treated MSCs exhibited the apoptotic characteristics of chromatin condensation and typical fragmented nuclei. Treatment with SB202190 efficiently blocked the apoptotic process and decreased the number of apoptotic cells. The degree of apoptosis induced was determined by monitoring positive Annexin V-FITC stained cells. As shown in Figure 1C,D, SB202190 significantly attenuated hypoxia/SD-induced Annexin V^+/PI^- accumulation. Taken together, these data indicate that hypoxia/SD-induced p38 activation contributes to hypoxia/SD-induced MSC apoptosis.

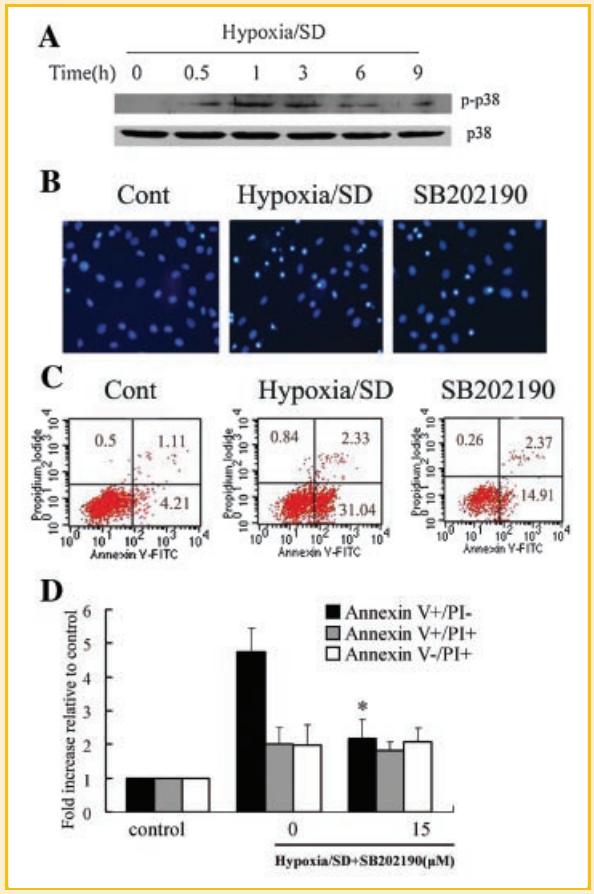

Fig. 1. Inhibition of p38 protects MSCs against hypoxia/SD-induced apoptosis. MSCs were treated with SB202190 as described in the Materials and Methods Section. A: Activation of p38 by Western blot analysis of the kinetics of p38 phosphorylation. B: A represented pattern of apoptotic nuclear condensation using fluorescence microscopy following Hoechst 33342 staining. C,D: Flow cytometric analysis of apoptotic cells after Annexin V-FITC and propidine iodide (PI) staining. Viable cells are Annexin V^-/PI^-. The Annexin V^+/PI^- cells are in the early apoptotic phase, whereas the Annexin V^+/PI^+ cells are in the late apoptotic phase. Necrotic cells are shown as Annexin V^-/PI^+. The results are presented as fold changes compared with corresponding control cells. Each column represents the mean ± SEM of three independent experiments, $^*P < 0.05$ versus hypoxia/SD treatment alone. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

HYPOXIA/SD INDUCES ER STRESS-ASSOCIATED CASPASE-12 CLEAVAGE AND CHOP EXPRESSION

Recent advances have highlighted the importance of ER stress in cell death processes [Ferri and Kroemer, 2001]. To investigate whether the ER stress is involved in hypoxia/SD-induced apoptosis of MSCs, the effects of hypoxia/SD on spliced XBP1 protein expression and eIF2α phosphorylation were examined by Western blot. Both spliced XBP1 and eIF2α phosphorylation in MSCs increased after hypoxia/SD exposure (Fig. 2A). Next, we investigated the transcriptional induction of ER-specific chaperone GRP78, transcriptional factor CHOP and procaspase-12 in cultured MSCs under hypoxia/SD treatment. As shown in Figure 2B, mRNA levels of CHOP and procaspase-12 were up-regulated in MSCs by exposure to hypoxia/SD for different time intervals, whereas GRP78 transcription was significantly downregulated, which is inconsistent with classical ER stress response. Finally, we examined the levels of caspase-12 fragments (indicators of caspase-12 activation) and CHOP protein, both of which are important mediators of ER stress-induced apoptosis. As shown in Figure 2C, procaspase-12 was activated resulting in the cleavage of procaspase-12 to smaller cleaved caspase-12, and the CHOP expression also increased. These results demonstrate that hypoxia/SD induces an ER stress response in MSCs. Furthermore, since hypoxia/SD-induced downregulation of GRP78 mRNA was not affected by SB202190 treatment in our study (data not shown), we next focused on identifying the relationship between p38 activation and ER stress-associated apoptotic pathways (procaspase-12 cleavage and CHOP expression).

P38-MEDIATED APOPTOSIS INVOLVES ER CASPASE-12 APOPTOTIC PATHWAY

To further characterize the mechanism by which the induced p38 activation promotes MSCs death, we explored whether the apoptosis mediated by p38 activation had a connection with the ER caspase-12 pathway. First, we evaluated the contribution of procaspase-12 cleavage to the process of MSCs apoptosis by treating the cells with the caspase-12 inhibitor Z-ATAD-FMK prior to hypoxia/SD treatment. As shown in Figure 3C, 2.5 μM Z-ATAD-FMK significantly inhibited hypoxia/SD-induced procaspase-12 cleavage. Furthermore, about 30% of the apoptotic cells (Annexin V^+/PI^-) were reversed by Z-ATAD-FMK (Fig. 3A,B). Next, we examined the effects of p38 inhibitor SB202190 on the procaspase-12 cleavage by Western blot. Our results showed that the cleavage of procaspase-12 to smaller cleaved caspase-12 was inhibited by SB202190 at 3 and 6 h after hypoxia/SD treatment (Fig. 3D). Collectively, these results indicate that hypoxia/SD-induced cleavage of procaspase-12 contributes to MSC apoptosis and acts as a downstream target of p38 activation.

P38-MEDIATED APOPTOSIS INVOLVES MITOCHONDRIAL PATHWAY

Having shown the involvement of p38 in hypoxia/SD-induced procaspase-12 cleavage, we next asked whether p38 activation lead to mitochondrial dysfunction. To this end, we first analyzed the kinetics of cytochrome *c* translocation between mitochondria and cytosol in the absence and presence of SB202190. Western blot analysis revealed that exposure of MSCs to hypoxia/SD induced an increase in cytosolic cytochrome *c* levels, and this was accompanied by a parallel decrease of cytochrome *c* in mitochondrial fraction, the effect partly inhibited by SB202190 (Fig. 4A). In parallel with the above studies, we examined whether p38 pathway influenced the loss of mitochondrial membrane potential ($\Delta\Psi m$) in MSCs subjected to hypoxia/SD. $\Delta\Psi m$ of MSCs was estimated by JC-1 staining and analyzed by flow cytometry. As shown in Figure 4B,C, the cell populations in the control groups were mainly localized in the higher $\Delta\Psi m$ region. After hypoxia/SD exposure, $\Delta\Psi m$ in MSCs significantly decreased. Hypoxia/SD-induced $\Delta\Psi m$ decrease was partly reversed by SB202190 but was not affected by Z-ATAD-FMK. Results above demonstrate that mitochondrial pathway and procaspase-12 cleavage act in parallel downstream of p38 activation.

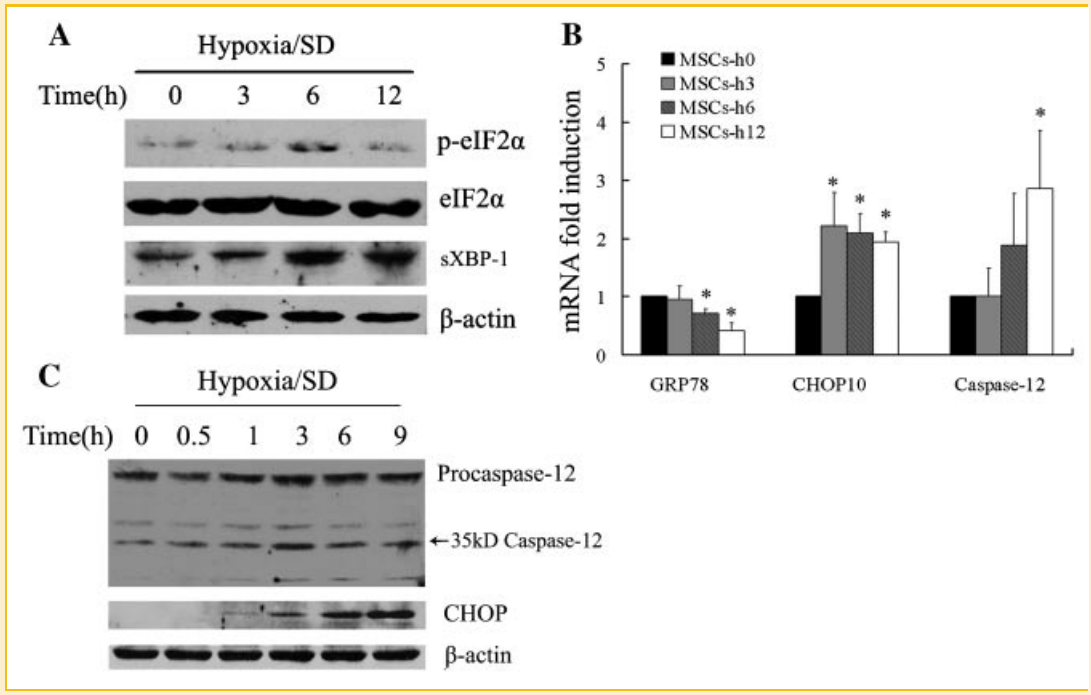

Fig. 2. Hypoxia/SD induces the ER stress response in MSCs. MSCs were incubated in hypoxia/SD for the indicated hours. A: Representative Western blots of p-eIF2α, eIF2α and spliced XBP-1. B: Relative mRNA levels of GRP78, CHOP and caspase-12 by real-time PCR analysis. The data in the graph are the mean ± SEM of at least three independent experiments. $^{*}P < 0.05$ versus control (MSCs-h0). C: Representative Western blots of procaspase-12 cleavage and CHOP expression.

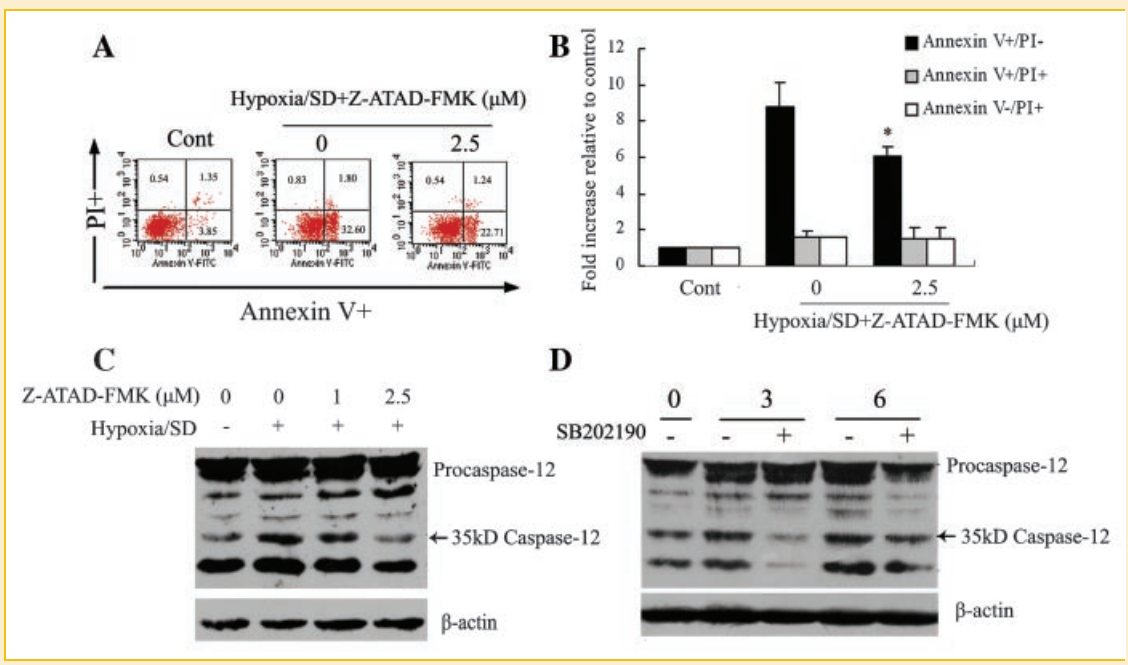

Fig. 3. Hypoxia/SD-induced p38 activation affects ER caspase-12 pathway. Caspase-12 inhibitor ZATAD-FMK (2.5 μM) was used in the same way as SB202190. A,B: Flow cytometric analysis of apoptotic cells after Annexin V-FITC and PI staining. Each column represents the mean ± SEM of three independent experiments, $^{*}P < 0.05$ versus hypoxia/SD treatment. C: Representative Western blots of procaspase-12 cleavage induced by hypoxia/SD and its inhibition by Z-ATAD-FMK. D: Representative Western blots of procaspase-12 cleavage induced by hypoxia/SD and its inhibition by SB202190. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

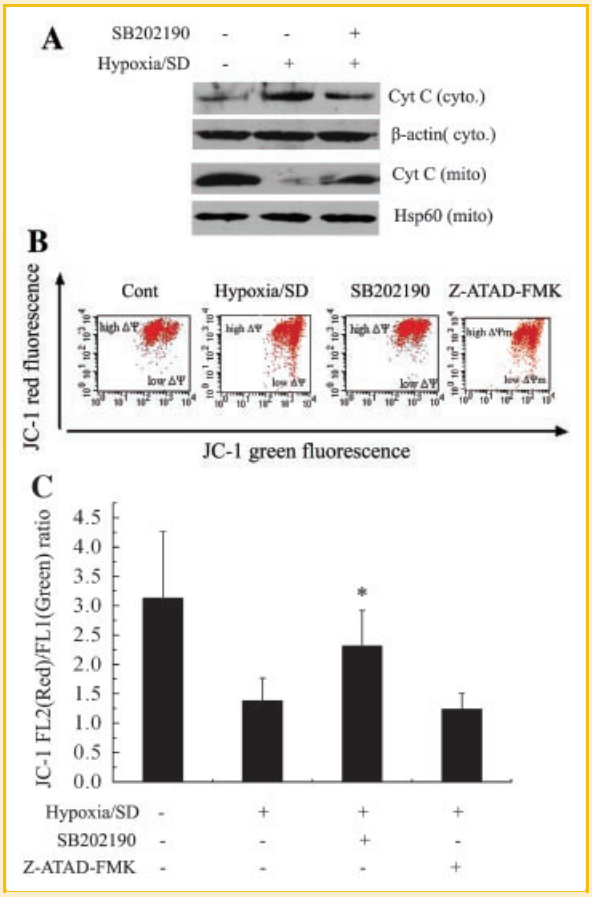

Fig. 4. Hypoxia/SD-induced p38 activation affects mitochondrial pathway. MSCs were treated with SB202190 as described in the Materials and Methods Section. A: Representative Western blots of translocation of cytochrome c between mitochondria and cytosol in the absence and presence of SB202190. B,C: Mitochondrial membrane potential ($\Delta\Psi$m) evaluated by staining with the potential sensor JC-1 and analyzed by flow cytometry. Cell populations with higher and lower JC-1 aggregated staining are marked with high $\Delta\Psi$ and low $\Delta\Psi$, respectively. A decrease in the bar indicates a shift in the fluorescence ratio correlating with an increase in mitochondrial depolarization. Each column represents the mean ± SEM of three independent experiments, $^{*}P < 0.05$ versus hypoxia/SD treatment. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

INHIBITION OF p38 ENHANCES HYPOXIA/SD-INDUCED CHOP EXPRESSION

Since hypoxia/SD-induced p38 activation mediated procaspase-12 cleavage, the role of p38 in the induction of CHOP expression, another important mediator of ER stress-induced apoptosis, was also examined. Unexpectedly, hypoxia/SD-induced CHOP expression was enhanced by p38 inhibitor SB202190 (Fig. 5A). Next, to clarify whether p38 inhibitor SB202190 affected the transcription of CHOP, the levels of CHOP mRNA in the absence and presence of SB202190 were detected by real-time PCR. As shown in Figure 5B, hypoxia/SD-induced CHOP transcription was also significantly enhanced by SB202190 treatment. The results above indicate that in hypoxia/SD-stimulated MSCs, the activation of p38 has an inhibitory effect on CHOP expression.

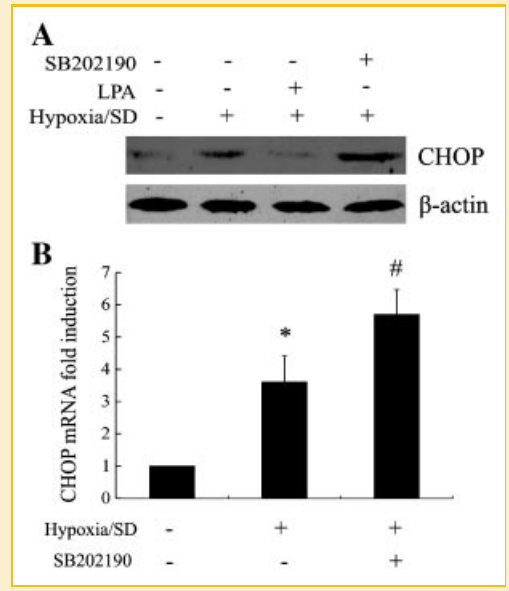

Fig. 5. P38 inhibition enhances hypoxia/SD-induced CHOP expression. MSCs were treated with SB202190 and LPA as described in the Materials and Methods Section. A: Representative Western blots of CHOP expression in the absence and presence of LPA and SB202190. B: Relative mRNA level of CHOP in the absence and presence of SB202190. $^{*}P < 0.05$ versus control; $^{\#}P < 0.05$ versus hypoxia/SD alone.

LPA INHIBITS p38 ACTIVATION, PROCASPASE-12 CLEAVAGE, AND CHOP EXPRESSION

Since LPA protected MSCs against hypoxia/SD-induced mitochondrial dysfunction [Chen et al., 2008], and p38 acted upstream of mitochondrial pathway, the effects of LPA on hypoxia/SD-induced p38 activation and its downstream procaspase-12 cleavage were therefore examined. As shown in Figure 6A, the level of phospho-p38 increased after treatment with hypoxia/SD for 6 h, whereas the effect was abrogated by LPA treatment. As expected, the cleavage of procaspase-12 was also inhibited by LPA, which is similar to the effects of SB202190 (Fig. 6B). However, unlike SB202190, LPA also inhibited hypoxia/SD-induced CHOP expression (Figs. 5A and 6B). Collectively, these observations indicate that LPA inhibits hypoxia/SD-induced p38 activation and its downstream procaspase-12 cleavage as well as CHOP expression.

LPA INHIBITS p38 ACTIVATION THROUGH $LPA_{1/3}$/Gi-LINKED ERK PATHWAY

We have previously shown that LPA protected MSCs from hypoxia/SD-induced apoptosis through the $LPA_{1/3}$/Gi-coupled PI3K/Akt and ERK pathways [Chen et al., 2008]. Therefore, the roles of $LPA_{1/3}$ and Gi protein on the inhibition of p38 by LPA were also examined. As shown in Figure 7A, 50 μM DGPP or 10 μM Ki16425 (LPA_1/LPA_3 antagonist) was able to reverse the inhibitory effects of LPA on p38 activation. Furthermore, preincubation of MSCs with the potent Gi protein inhibitor PTX also reversed the inhibitory effects of LPA.

To further explore the signaling pathways through which LPA impairs hypoxia/SD-induced p38 activation, we first used 100 nM wortmannin to inhibit PI3K/Akt pathway in MSCs, and then the

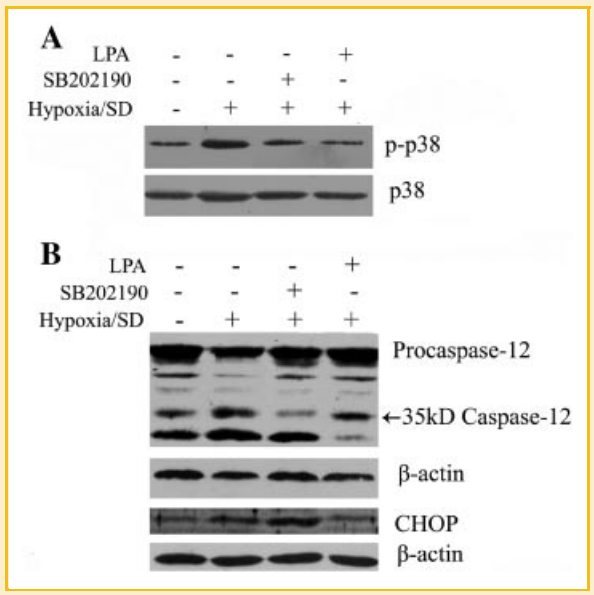

Fig. 6. LPA inhibits p38 activation, procaspase-12 cleavage and CHOP expression. MSCs were treated with SB202190 and LPA as described in the Materials and Methods Section. A: Representative Western blots of phospho-p38 and total p38 in the absence and presence of SB202190 and LPA. B: Representative Western blots of procaspase-12 cleavage and CHOP expression in the absence and presence of SB202190 and LPA.

levels of phospho-Akt, phospho-p38 and p38 in the absence and presence of LPA were detected by Western blot. As shown in Figure 7B, LPA could efficiently reverse hypoxia/SD-induced Akt inactivation, and wortmannin blocked this Akt promoting effect of LPA. However, neither the activation of p38 by hypoxia/SD nor the inhibition of p38 by LPA was affected by wortmannin. Next, we used 20 μM U0126 to inhibit LPA-stimulated ERK1/2 pathway in MSCs, and examined the levels of phospho-p38 and p38 by Western blot. As shown in Figure 7C, the inhibitory effect of LPA on p38 activation was released by U0126. These results indicate that it is $LPA_{1/3}$/Gi/ERK pathway but not PI3K/Akt pathway that mediates the inhibitory action of LPA on p38 activation.

P38 INHIBITION BY LPA MAY BE DEPENDENT ON ERK-MEDIATED MKP-1 INDUCTION

Since LPA inhibits hypoxia/SD-induced p38 activation, we next investigated the changes of MKP-1 expression, a critical negative regulator of p38, in response to hypoxia/SD. As shown in Figure 8A, rapid downregulated mRNA of MKP-1 occurred after 3 h of hypoxia/SD, with a slight increase up to 9 h. Since LPA can induce the expression of MKP-1 through ERK-dependent and Ca^{2+}-dependent signals in Rat-1 cells [Cook et al., 1997], we postulated that the inhibition of p38 by LPA may be caused by the induction of MKP-1. To confirm this, we used 20 μM U0126 to inhibit ERK1/2 pathway in MSCs, and then the mRNA and protein levels of MKP-1 in the absence and presence of LPA were measured. The results showed that LPA could partly reverse hypoxia/SD-induced downregulation of MKP-1 mRNA (Fig. 8B) and protein (Fig. 8C), the effect abolished by inhibition of ERK1/2 pathway with U0126. Collectively, these

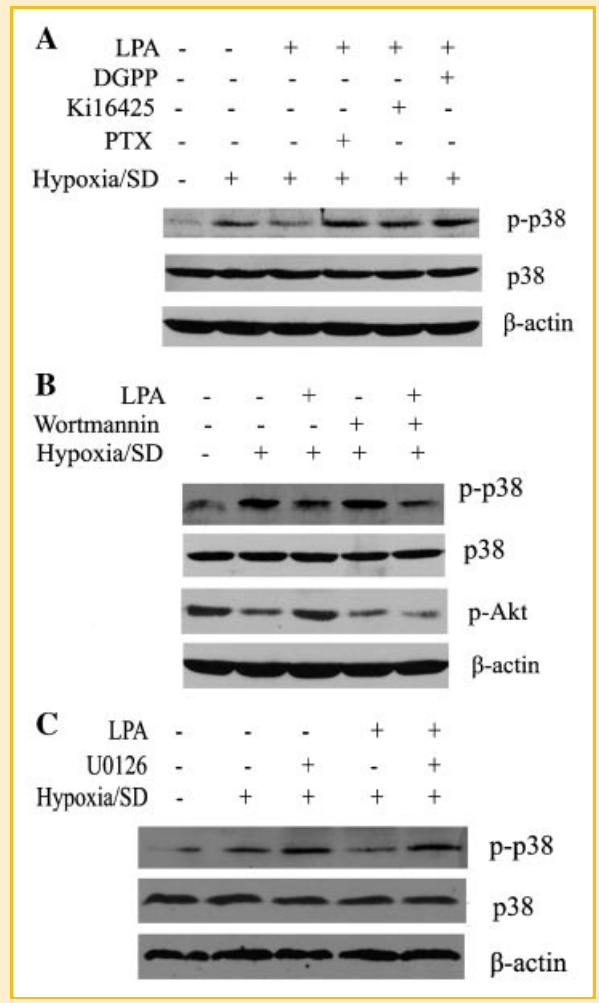

Fig. 7. LPA inhibits p38 activation through $LPA_{1/3}$/Gi/ERK pathway. MSCs were pretreated with either PTX (200 ng/ml) for 16 h or DGPP (50 μM), Ki16425 (10 μM), U0126 (20 μM), or Wortmannin (100 nM) for 80 min before exposure to hypoxia/SD for 6 h. LPA (10 μM) was added in the presence of each drug for 1 h prior to exposure to hypoxia/SD. All drugs were maintained in the incubation medium throughout the hypoxia/SD treatment period. A–C: Representative Western blot of phospho-p38 and total p38 in the absence and presence of the different drugs listed above.

findings suggest that the inhibitory effects of LPA on hypoxia/SD-induced p38 activation may be dependent on $LPA_{1/3}$/Gi/ERK-mediated MKP-1 induction.

DISCUSSION

MSCs transplantation therapy has attracted considerable interest in recent years. However, the biggest dilemma faced is that most of the transplanted MSCs do not fully establish and are readily lost, presumably due to cell death induced by the ischemic environment into which they are introduced in vivo [Geng, 2003; Mangi et al., 2003; Saito et al., 2003]. Our previous study demonstrated that hypoxia/SD induced programmed MSCs death through the

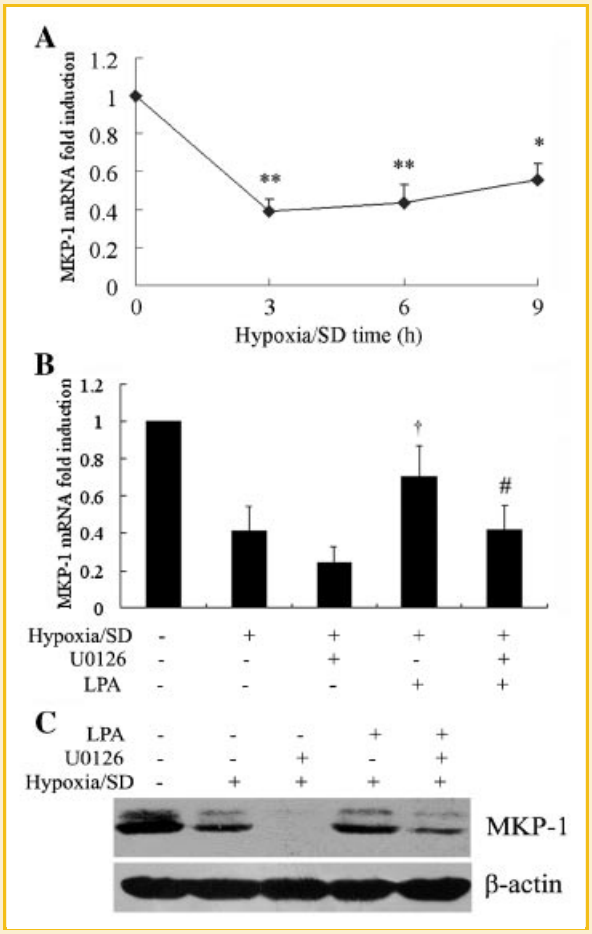

Fig. 8. The induction of MKP-1 transcription by LPA is dependent on the ERK pathway. MSCs were pretreated with U0126 (20 μM) for 80 min before exposure to hypoxia/SD for 6 h. LPA (10 μM) was added in the presence of U0126 for 1 h prior to exposure to hypoxia/SD. A: Relative mRNA levels of MKP-1 in MSCs induced by hypoxia/SD for the indicated time intervals. Each data point represents the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus normal control; (B) shows relative mRNA levels of MKP-1 in MSCs induced by hypoxia/SD for 6 h in the absence and presence of U0126 and LPA. Data are shown as mean ± SEM. $^{\dagger}P < 0.05$ versus hypoxia/SD alone; $^{\#}P < 0.05$ versus LPA treatment. C: Representative Western blot of MKP-1 in MSCs induced by hypoxia/SD for 6 h in the absence and presence of U0126 and LPA.

mitochondrial apoptotic pathway [Zhu et al., 2006]. Since ER stress is also an important factor of ischemia-related apoptosis [Tajiri et al., 2004; Azfer et al., 2006; Rissanen et al., 2006], we are interested in identifying whether ER stress is involved in hypoxia/SD-induced MSCs apoptosis.

After hypoxia/SD treatment, we observed marked induction of CHOP transcription, spliced XBP-1 expression, eIF2α phosphorylation as well as procaspase-12 cleavage and CHOP expression in MSCs. Inhibition of procaspase-12 cleavage could partly reverse hypoxia/SD-induced MSC apoptosis. These findings indicate that procaspase-12 cleavage is involved in hypoxia/SD-induced MSCs apoptosis. To our knowledge, our study is the first to report the involvement of ER stress-associated caspase-12 in hypoxia/SD-induced MSCs apoptosis.

LPA is the smallest and structurally simplest glycerophospholipid, which accumulates in patients with acute myocardial infarction, increasing more than sixfold 48–72 h after the onset of the disease [Chen et al., 2003]. Furthermore, LPA can block the mitochondrial pathway, thus protecting MSCs from hypoxia/SD-induced apoptosis [Chen et al., 2008]. The present study demonstrated that LPA also attenuated procaspase-12 cleavage and CHOP expression in hypoxia/SD-stimulated MSCs, suggesting the ER stress-associated LPA targets to antagonize MSCs apoptosis induced by hypoxia/SD.

The p38 activity has been reported to be associated with apoptotic induction in several cell types in response to a multitude of cellular stresses [Porras et al., 2004], and p38-mediated apoptosis involves different pathways [Cai and Xia, 2008; Kim and Chung, 2008; Lu et al., 2008; Yamagishi et al., 2008]. The present study demonstrated that hypoxia/SD-induced p38 activation not only mediated the mitochondrial pathway by induction of cytochrome *c* release but also mediated the ER caspase-12 apoptotic pathway. Interestingly, the induced p38 activation has an inhibitory effect on hypoxia/SD-induced CHOP expression, based on the fact that inhibition of p38 by SB202190 enhanced hypoxia/SD-induced CHOP expression. The inhibitory effects of SB202190 and LPA on p38 phosphorylation are similar (Fig. 6A), but the anti-apoptotic effect of LPA is more striking than that of SB202190. This may be due to the enhanced CHOP expression by SB202190 (Fig. 5A). Thus we hypothesize that hypoxia/SD-induced p38 activation may play pro-apoptotic and anti-apoptotic dual roles, just as previously reported [Birkenkamp et al., 1999; Nakagawa et al., 2004].

LPA can activate p38, mediating a broad range of biological processes [Malchinkhuu et al., 2005; Saatian et al., 2006; Kim et al., 2008]. However, our current study demonstrates that in the context of hypoxia/SD, LPA abrogates p38 activation in MSCs. The exact reason for the differential regulation of p38 by LPA is not clear, but there are at least two possibilities. One possibility may be the difference in the cell type used in the experiments. Baudhuin et al. [2002] have used 12 cell lines to test the cellular response to LPA, and found the regulation of p38 by LPA was not completely identical, although 11 cell lines showed the common action of LPA on p38. Each cell type may have distinct structural characteristics and diverse distribution of LPA receptor subtypes and the receptor abundance. The other concerning possibility is the variation of experimental conditions which might account for the different cellular responses to LPA. All the evidence that LPA activates p38 is based on the normal culture conditions. However, in our study, hypoxia/SD may induce lots of pathological changes which may interfere with LPA function. Thus, the opposite regulation of p38 by LPA may be an interesting topic for further investigation.

Our previous study has shown that LPA-mediated protection against hypoxia/SD-induced apoptosis in MSCs is dependent on both ERK1/2 and PI3K/Akt signaling pathways, which act in parallel and downstream of $LPA_{1/3}$/Gi pathway [Chen et al., 2008]. Our current study further demonstrates that inhibition of hypoxia/SD-induced p38 by LPA is also dependent on $LPA_{1/3}$/Gi pathway. Moreover, the inhibitory effect of LPA is dependent on the ERK1/2 pathway but independent of the PI3K/Akt pathway. Since the genetic modification of MSCs with Akt increases the post-transplantation

viability of these cells and enhances their therapeutic efficacy [Mangi et al., 2003], the ERK1/2 pathway may be another molecular target to increase the viability of MSCs transplanted into the ischemic heart.

In light of the important role of p38 in hypoxia/SD-induced MSC apoptosis and the importance of protein kinase/phosphatase balance in phosphorylation-dependent signaling pathways, the regulatory role of phosphatase in apoptosis has attracted our attention. MKP-1 plays a pivotal role in the deactivation of p38, and MKP-1 deficiency results in enhanced p38 activation [Bueno et al., 2001; Wu and Bennett, 2005; Zhao et al., 2005]. The results of our present study demonstrate that hypoxia/SD downregulated MKP-1 expression in MSCs, while this process was partly reversed by LPA. Furthermore, consistent with previous findings [Cook et al., 1997], blocking ERK1/2 activity abolished the action of LPA on MKP-1 induction. Our data suggest that the protective role of LPA may be at least partly mediated by the $LPA_{1/3}$/Gi/ERK/MKP-1 pathway, which results in the inhibition of p38 activation and its downstream mitochondrial and ER caspase-12 apoptotic pathways. Based on the present results, we suspect that p38 activity is negatively regulated by MKP-1 in normal MSCs and kept in a low activity state. In the context of hypoxia/SD, the downregulation of MKP-1 leads to p38 activation and induces the apoptotic process, while LPA attenuates hypoxia/SD-induced MKP-1 downregulation, resulting in the inhibition of p38-mediated apoptosis. Importantly, the regulation of MKP-1 might be an efficient strategy to elevate the viability of transplanted MSCs.

In summary, the results obtained in the present study indicate that in addition to the previously reported mitochondrial pathway, the ER caspase-12 apoptotic pathway is also involved in hypoxia/SD-induced apoptosis of MSCs. Hypoxia/SD induces p38 activation, which acts upstream of mitochondrial and ER procaspase-12 apoptotic pathways. LPA inhibits both of these apoptotic pathways through inhibition of p38 activation, which is dependent on $LPA_{1/3}$/Gi-linked ERK1/2 pathway but independent of the PI3K/Akt pathway. Furthermore, $LPA_{1/3}$/Gi/ERK pathway-mediated MKP-1 induction may account for the inhibition of p38 by LPA. Our findings may provide the molecular basis for elevating the viability of MSCs transplanted into ischemic myocardium for therapeutic purposes.

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