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Lovastatin Protects Mesenchymal Stem Cells Against Hypoxia- and Serum Deprivation-Induced Apoptosis by Activation of PI3K/Akt and ERK1/2

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Abstract Cell therapy with bone marrow-derived mesenchymal stem cells (MSCs) has been shown to have great promises in cardiac repair after myocardial infarction. However, poor viability of transplanted MSCs in the infracted heart has limited the therapeutic efficacy. Our previous studies have shown in vitro that rat MSCs undergo caspase-dependent apoptosis in response to hypoxia and serum deprivation (Hypoxia/SD). Recent findings have implicated statins, an established class of cholesterol-lowering drugs, enhance the survival of cells under various conditions. In this study, we investigated the effect of lovastatin on rat MSCs apoptosis induced by Hypoxia/SD, focusing in particular on regulation of mitochondrial apoptotic pathway and the survival signaling pathways. We demonstrated that lovastatin (0.01–1 μM) remarkably prevented MSCs from Hypoxia/SD-induced apoptosis through inhibition of the mitochondrial apoptotic pathway, leading to attenuation of caspase-3 activation. The loss of mitochondrial membrane potential and cytochrome-*c* release from mitochondria to cytosol were significantly inhibited by lovastatin. Furthermore, the antiapoptotic effect of lovastatin on mitochondrial apoptotic pathway was effectively abrogated by both PI3K inhibitor, LY294002 and ERK1/2 inhibitor, U0126. The phosphorylations of Akt/GSK3β and ERK1/2 stimulated by lovastatin were detected. The activation of ERK1/2 was inhibited by a PI3K inhibitor, LY294002, but U0126, a ERK1/2 inhibitor did not inhibit phosphorylation of Akt and GSK3β. These data demonstrate that lovastatin protects MSCs from Hypoxia/SD-induced apoptosis via PI3K/Akt and MEK/ERK1/2 pathways, suggesting that it may prove a useful therapeutic adjunct for transplanting MSCs into damaged heart after myocardial infarction. J. Cell. Biochem. 103: 256–269, 2008.

Key words: lovastatin; mesenchymal stem cells; survival; apoptosis; signaling pathway

Myocardial infarction leads to the permanent loss of cardiomyocytes, followed by pathological left ventricle remodeling and progression to heart failure. Although some reports have shown evidence of mitotic division of cardiomyocytes after myocardial infarction [Beltrami et al., 2001], the mitotic capacity of heart muscle is too limited to fully substitute for cells lost following myocardial infarction. The goals of cellular cardiomyoplasty are to replace cardiomyocytes lost after ischemia, induce revascularization of the injured region, and prevent deleterious pathological remodeling after myocardial infarction [Hamano et al., 2002; Toma et al., 2002]. A large number of studies have recently demonstrated that cell therapy with bone marrow mesenchymal stem cells (MSCs) has great promises in regenerating and repopulating the damaged myocardium, restoring its function and is a safe effective strategy for treating ischemic heart failure [Stamm et al., 2003; Katritsis et al., 2005; Mark et al., 2006; Miyahara et al., 2006]. However, the efficacy of this approach is limited by poor viability of MSCs transplanted into the infracted heart [Mangi et al., 2003; Kenichiro et al., 2005; Tang et al., 2005]. For example, more than 99% of MCSs injected into the left ventricle of CB17 SCID/beige adult mice died within 4 days of injection [Geng, 2003], implying that the

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ischemic microenviroment of the infracted myocardium could not be conducive of MSCs survival. Moreover, our previous study has demonstrated that MSCs undergo caspase-dependent apoptosis induced by hypoxia and serum deprivation (Hypoxia/SD) in which mitochondrial dysfunction is involved [Zhu et al., 2006]. Therefore, promoting the survival of implanted MSCs is important for the efficacy of stem cell therapy. To our knowledge, there is little information available in literature about investigating a strategy to protect the MSCs against apoptosis.

Hydroxymethylglutaryl-coenzyme A (HMGCoA) reductase inhibitors, known as "statins," have been highly effective for lowering serum cholesterol and reducing the incidence of coronary events [Calabro and Yeh, 2005]. Interest is emerging in the so-called pleiotropic effects of statins on cardiovascular diseases, such as inhibiting proinflammatory cytokine activity, reducing cardiomyocyte apoptosis, improving endothelial function, and increasing immunotolerance after transplantation [Endres, 2006; Hong et al., 2006]. Increasing amount of evidence implies that statin act as an antiapoptotic role in a variety of cells. For example, statins improve cardiac function in rats with aortic stenosis by inhibiting cadiomyocyte apoptosis [Son et al., 2006], protect human aortic smooth muscle cells from calcication via inhibiting apoptosis [Chen et al., 2004], preserve the cardiomyocytes mitochondrial function challenging with H_2O_2 in vitro [Jones et al., 2003], and inhibit β-adrenergic receptor-stimulated apoptosis in adult rat ventricular myocytes [Masahiro et al., 2004]. And also statins are protective against ischemia-reperfusion cardiac injury and reduce apoptosis of cardiomyocytes in pigs and rats [Di Napoli et al., 1999; Dworakowski et al., 2006]. Despite these above antiaopototic effects of statins were reported, however, it is unclear whether statins may protect MSCs from apoptosis under conditions of Hypoxia/SD.

Both phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK1/2) pathways play important roles in mediating survival signaling [Zhan and Han, 2004; Maria et al., 2005; Risbud et al., 2005]. The aim of this study was to investigate whether lovastatin can inhibit apoptosis of mesenchymal stem cells subjected to hypoxia and serum deprivation, and to further examine the putative role of PI3K/Akt and MEK/ERK1/2 in lovastatin-mediated effects.

MATERIALS AND METHODS

Materials

Iscove's modified Dulbecco's medium (IMDM) and fetal bovine serum (FBS) were from Gibco (Grand Island, NY). LY294002, U0126, rabbit polyclonal antibodies, anti-phospho-p44/42 (Thr202/tyr204), anti-p44/42, anti-phospho-Akt (ser-473), anti-Akt, anti-caspase-3, and anti-phospho-GSK3β (ser9) (Cell Signal Technology, Beverly, MA); mouse monoclonal antibodies, anti-β-actin (SantaCruz), anti-GSK3β (KangChen Bio-Tech, KangChen, SH). Horseradish peroxidase-conjugated secondary antibodies to mouse or rabbit were obtained from Santa Cruz Biotechnology. Caspase-3/CPP32 colorimetric assay kit and cytochrome-*c* releasing apoptosis assay kit were purchased from Biovision (Biovision Research, CA).

Cell Culture

The MSCs were prepared from Sprague–Dawley rats and grown in IMDM with 15% (v/v) FBS and 100 U/ml penicillin–streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Cells were used at first passage for all experiments. MSCs apoptosis was induced by Hypoxia/SD for 6 h as previously described [Zhu et al., 2006]. For lovastatin protection experiments, MSCs were pretreated with lovastatin (0.001–1 μM) for 1 h and continuously for 6 h of hypoxia and serum deprivation. For inhibitor studies, the cells were preincubated with a PI3K inhibitor LY294002 (25 μM) or MEK1/2 inhibitor U0126 (10 μM) before the addition of lovastatin (1 μM).

Assessment of Morphological Changes

Chromosomal condensation was assessed using the chromatin dye Hoechst 33342 (Sigma–Aldrich, St. Louis, MO). Cells were fixed at room temperature for 30 min in phosphate-buffered saline (PBS) containing 1% glutaraldehyde, washed twice with PBS and then exposed to 5 μg/ml Hoechst 33342 in PBS for 30 min at room temperature. All samples were observed using a phase contrast microscope and a fluorescence microscope for apoptotic cells, which were characterized by

morphological alteration such as condensed nuclei and cell shrinkage.

Measurement of Cell Apoptosis and Mitochondrial Membrane Potential

Apoptosis was assessed using an Annexin V-FITC/PI binding assay. Flow cytometric analysis of Annexin V-FITC and PI-stained cells was performed using an apoptosis detection kit (Oncogene, San Diego) according to the manufacturer's instructions. Briefly, cells were rinsed with ice-cold PBS and then resuspended in 200 μl of binding buffer. Ten microliters of Annexin V stock solution was added to the cells and incubated for 30 min at 4°C. The cells were then further incubated with 5 μl propidium iodide (PI) and immediately analyzed using a FACScan. Ten thousand events were acquired on a FACSC-LSR (Becton-Dickinson, San Jose, CA) and analyzed with CellQuest (Becton-Dickinson) software.

The mitochondrial transmembrane potential ($\triangle\Psi$m) was analyzed using $\triangle\Psi$m-specific stain Rhodamine 123 (Sigma–Aldrich). In brief, in each of the sample, approximately 10^5 cells were stained in a solution containing 0.1 μM Rhodamine 123 for 30 min at 37°C. Staining was quantified by scatter characteristic using a flow cytometer EPICS XL from Beckman Coulter (Fullerton, CA).

Preparation of Cell Lysates

For each assay, 5×10^5 cells were incubated with various concentrations of lovastatin before hypoxia and serum deprivation. The cells were harvested and centrifuged at $500 \times g$ for 10 min at 4°C. The pellets were resuspended in lysis buffer containing 1%Triton X-100, 20 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethanesulfonylfluoride, and 1 mg/ml each of leupeptin, aprotinin, and pepstatin for 30 min. The lysates were centrifuged at $14{,}000 \times g$ for 10 min at 4°C The supernatants were frozen at −70°C. Protein concentration of the supernatants was quantified with the Bradford Protein assay.

Western Blot Analysis

For analysis of protein levels, the supernatants were then mixed with 5 × SDS sample buffer, boiled for 5 min, and separated through 8% to 15% SDS–PAGE gels. After electrophoresis, the proteins were transferred to nylon membranes by electrophoretic transfer. Nonspecific binding was blocked with 5% skim milk for 2 h, rinsed, and incubated overnight at 4°C with primary antibody in 5% skim milk. The membrane was washed in TBS/0.1% Tween 20, and incubated for 2 h with horseradish peroxidase-conjugated secondary antibody. After washes in TBS/0.1% Tween 20, bands were visualized by enhanced chemoluminescence and exposed to radiography film.

For preparation of mitochondrial and cytosolic fractions, a cytochrome-*c* releasing apoptosis assay kit was used. Briefly, cells were harvested and washed twice with ice-cold PBS, then the highly enriched mitochondria fraction was isolated from the cytosolic fraction according to the formulations in the assay kit. Cytochrome-*c* released into the cytosol from mitochondria and that in mitochondria were then respectively determined by Western blotting using the cytochrome-*c* antibody provided in the kit.

Determination of Caspase-3 Activity

The caspase-3 activity was determined using Caspas-3/CPP32 colorimetric assay kit according to the manufacturer's protocol. Briefly, protein extracts (150 g) were incubated for 60 min at 37°C in 100 mM HEPES containing 10% sucrose, 0.1% CHAPS, 1 mM EDTA, 10 mM DTT, pepstatin (10 μg/ml), and leupeptin (10 μg/ml) with the substrate DEVD-pNA (200 μM final concentration) in a total volume of 100 μl. The pNA light emission after cleavage from the substrate was quantified using an absorption spectrophotometer at 406 nm (Bio-Rad).

Statistics

Data are expressed as mean ± standard deviation. 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

Lovastatin Prevented Apoptosis of MSCs Induced by Hypoxia/SD

Previous studies have shown that Hypoxia/SD triggered apoptotic MSCS death by nuclear shrinkage, chromatin condensation, and decrease in cell size. The MSCs were exposed to

culture conditions represented by Hypoxia/SD with or without lovastatin at different concentrations and characteristics of cell apoptosis were analyzed by morphology and flow cytometry. Cells exposed to Hypoxia/SD had increased chromatin condensation and were decreased in cell size compared to cells maintained in normoxic (normoxic, IMDM with 15% FBS) control conditions (Fig. 1A,B). Cells treated with lovastatin at concentrations of 0.01, 0.1,

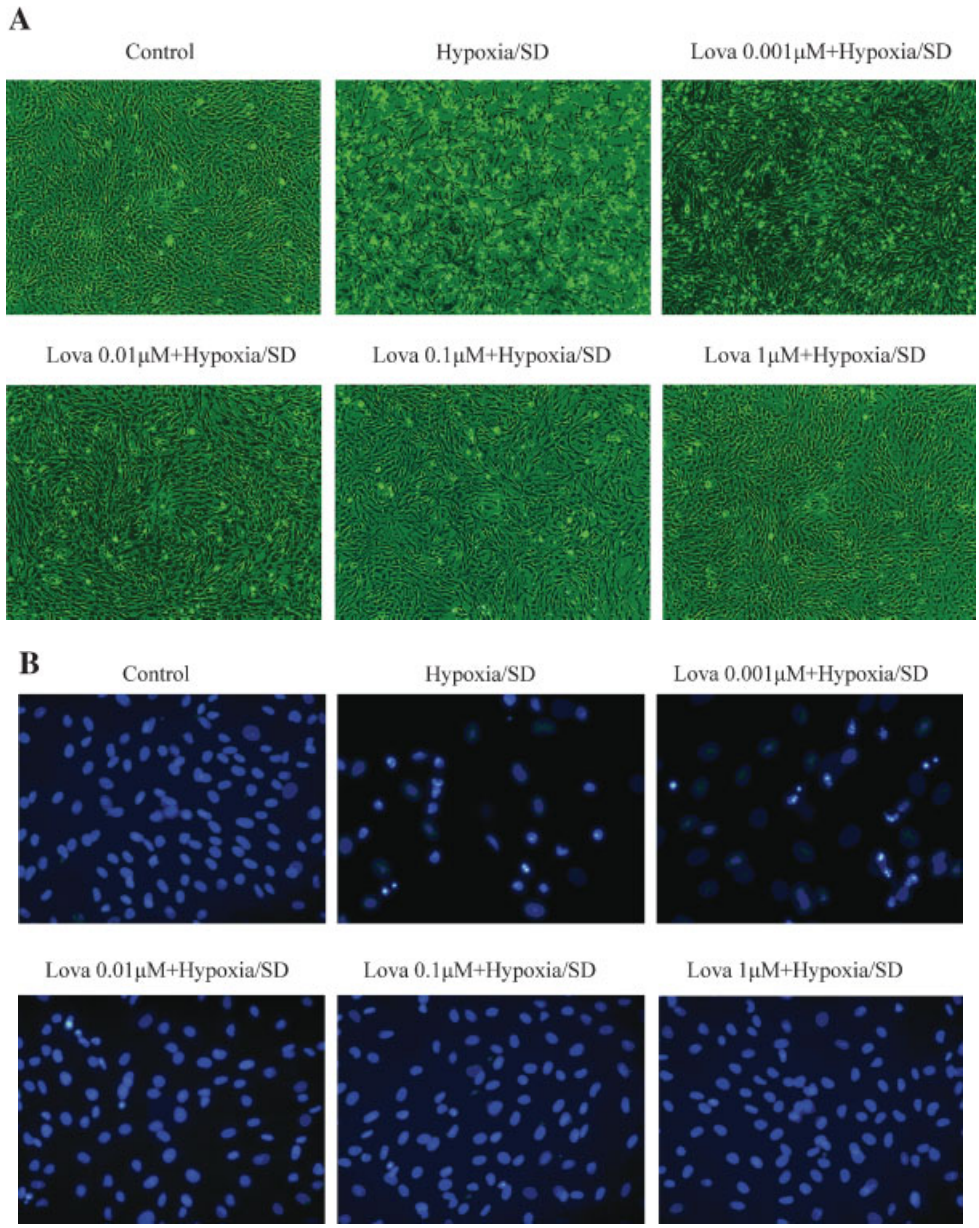

Fig. 1. Lovastatin protects mesenchymal stem cells (MSCs) against hypoxia and serum deprivation (Hypoxia/SD)-induced apoptosis. Lovastatin (Lova) at concentrations of 0.001 μM, 0.01 μM, 0.1 μM, and 1 μM (final concentration) were applied to MSCs 1 h before subjected to Hypoxia/SD. The antiapoptotic effects of lovastatin were then determined by detection of NSCs with morphological Changes using phase-contrast microscope (**A**) and with apoptotic nuclear morphology using fluorescence microscopy upon Hoechst 33342 staining (**B**). **C**, **D**: Apoptosis was then quantified by FACS analysis after staining with Annexin V and propidine iodine (PI). Viable cells are Annexin V−/PI−. The Annexin V+/PI− cells are early in the apoptotic process, whereas the Annexin V+/PI+ cells indicating late apoptosis have lost cell membrane integrity and have taken up PI. Necrotic cells show Annexin V−/PI+. The results are presented as fold changes compared with corresponding control cells or Hypoxia/SD cells. Each data point represents mean ± standard deviation of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control. $^{\#\#}P < 0.01$ compared with Hypoxia/SD cells.

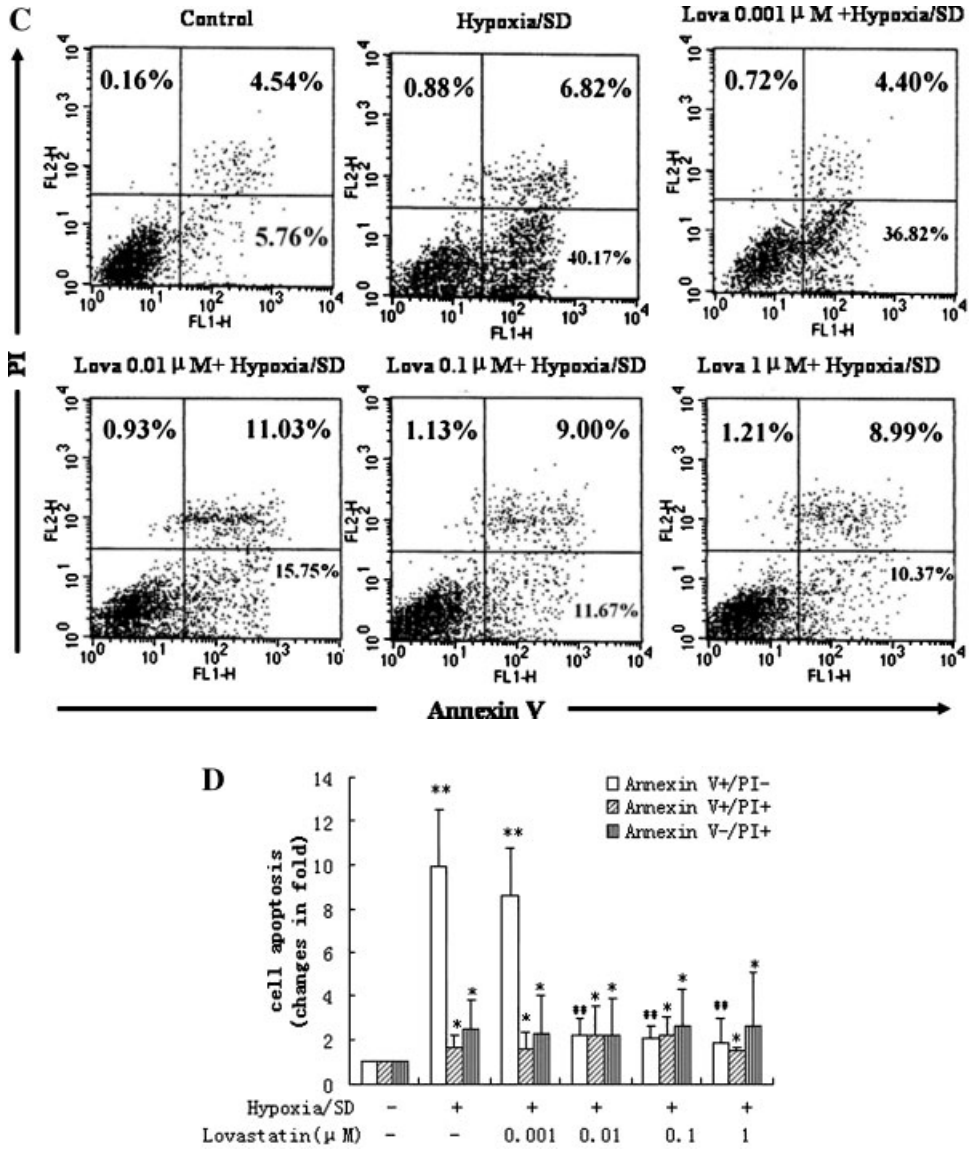

Fig. 1. (*Continued*)

and 1 μM before Hypoxia/SD had large, regular nuclei, with relatively few showing the characteristic condensed chromatin and reduced size of apoptotic cells. Cell death was also measured using cell binding of the fluorescent dye Annexin V-FITC, which marks the early stage membrane change in apoptosis and PI staining of the nucleus, which marks late apoptotic events and necrosis. Figure 1C,D shows the percentages of Annexin V-stained and PI-stained cells in response to Hypoxia/SD and lovastatin treatment. It was observed that lovastatin (0.01–1 μM) had significant survival effects on early phase (Annexin V+/PI−) as compared with the Hypoxia/SD group ($P < 0.01$), but had no effects on late phase (Annexin V+/PI+) or necrosis (Annexin V−/PI+) MSCs. We also observed that a concentration of 0.001 μM had no antiapoptotic effect on the MSCs induced by Hypoxia/SD.

Lovastatin Inhibited Activation of Caspase-3 in Hypoxia/SD-Induced MSCs Apoptosis

Caspase-3 is a key effector of the aspartate-specific cysteine protease family that is thought to be a kry effector of cell apoptosis. For further evaluating the protective effect of lovastatin on MSCs, we tested the caspase-3 activity. Hypoxia/SD induced a cleavage of the 32-kDa pro-caspase-3, resulting in the appearance of

the 19-kDa active form of this caspase. Pretreatment with lovastatin at concentrations of 0.01 μM, 0.1 μM, and 1 μM, attenuated the Hypoxia/SD-induced formation of the 19-kDa active caspase (Fig. 2A). We also monitored caspase-3 activity with the substrate DEVD and found that lovastatin significantly reduced caspase-3 activity in MSCs induced by Hypoxia/SD ($P < 0.05$) (Fig. 2B), which confirmed the Western blot analysis of caspase-3 activation.

Lovastatin Protected Mitochondrial Function in Hypoxia/SD-Induced MSCs Apoptosis

Loss of mitochondrial membrane potential and permeability transition pore opening indicate the active role of mitochondria during the early phase of apoptosis [Korsnes et al., 2006].

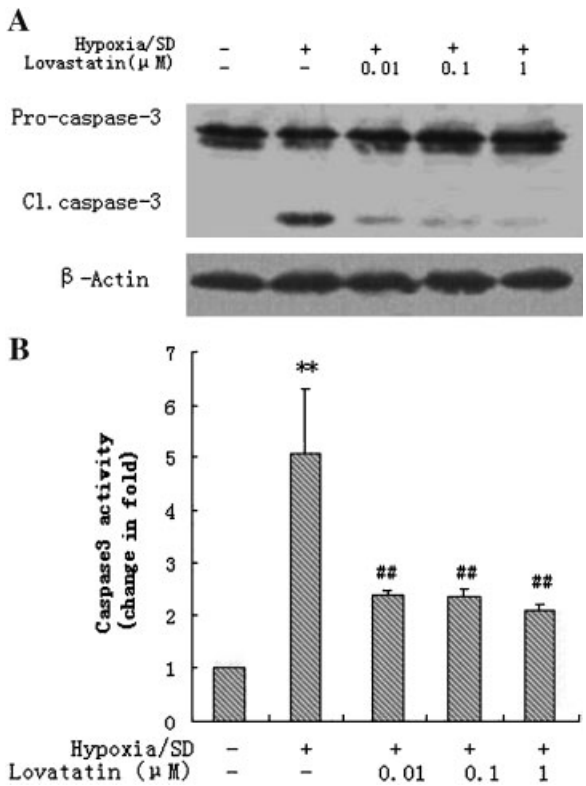

Fig. 2. Lovastatin protects MSCs against Hypoxia/SD-induced apoptosis by inhibiting caspase-3 activation. Lovastatin (Lova) at concentrations of 0.01 μM, 0.1 μM, and 1 μM (final concentration) were applied to MSCs 1 h before incubated in Hypoxia/SD. Analysis for procaspase-3 cleavage detected by Western blot with anti-procaspse and anti-cleaved caspase-3 (Cl. Caspase-3) antibody (**A**) and active caspase-3 determined by using Caspase-3/CPP32 Colorimetric Assay Kit (**B**). Activation level of caspase-3 is shown as a fold of change representing mean ± standard deviation of at least three independent experiments. $^{**}P < 0.01$ compared with control cells. $^{\#\#}P < 0.01$ compared with Hypoxia/SD cells.

Previously studies showed that Hypoxia/SD-induced cell apoptosis was associated with a loss in MSCS $\triangle\Psi$m [Zhu et al., 2006]. In this study, we assessed whether lovastatin enhanced mitochondrial function by maintaining $\triangle\Psi$m. In contrast to Hypoxia/SD group, cells treated with lovastatin at concentrations of 0.01, 0.1, and 1 μM prevented the fall in $\triangle\Psi$m (Fig. 3A,B).

Loss of mitochondrial membrane integrity contributes to the release of cytochrome-*c*. To investigate whether lovasatin prevented cytochrome-*c* release from mitochondria, we performed a fractionation of MSCs. And then the mitochondrial and cytosolic fractions were subjected to Western blot analysis. Our results demonstrated that lovastatin inhibited cytochrome-*c* release induced by Hypoxia/SD and this decrease of cytochrome-*c* in cytosolic correlated with its increase in the mitochondrial fraction (Fig. 3C).

These results suggested that lovastatin preserved the mitochondrial function by maintaining the $\triangle\Psi$m and inhibiting release of cytochrome-*c* in Hypoxia/SD-induced MSCs apoptosis. Above results indicated that lovastatin at concentrations of 0.01 μM, 0.1 μM, and 1 μM had equalpotent effects. In the following experiments, a concentration of 1 μM was chosen for detection.

Lovastatin-Activated PI3K/Akt Pathways

We next examined the possible involvement of PI3K/Akt pathway in lovastatin-mediated antiapoptotic effects. The apoptotic population was measured by flow cytometric analysis. As shown in Figure 4A,B an increase of apoptotic cells was observed after induction of Hypoxia/SD ($P < 0.01$), but the MSCs treated with lovastatin exhibited less staining with Annexin V^+/PI^- than that of Hypoxia/SD group ($P < 0.01$). As expected, the antiapoptotic action of Lovastatin was abolished by the PI3K inhibitor LY294002, indicating a PI3K-mediated mechanism.

To further establish a role for the PI3K pathway in the antiapoptotic effect of lovastatin, the activation status of Akt was determined. After serum-starvation for 12 h, MSCs were stimulated with lovastatin (1 μM) and cells were harvested at 0, 5, 10, 20, 30, 40, and 60 min respectively. Activation of Akt was analyzed by Western blot using anti-phospho-Akt antibody. Exposure of MSCs to lovastatin stimulated Akt activity, and the level of phosphorylation was

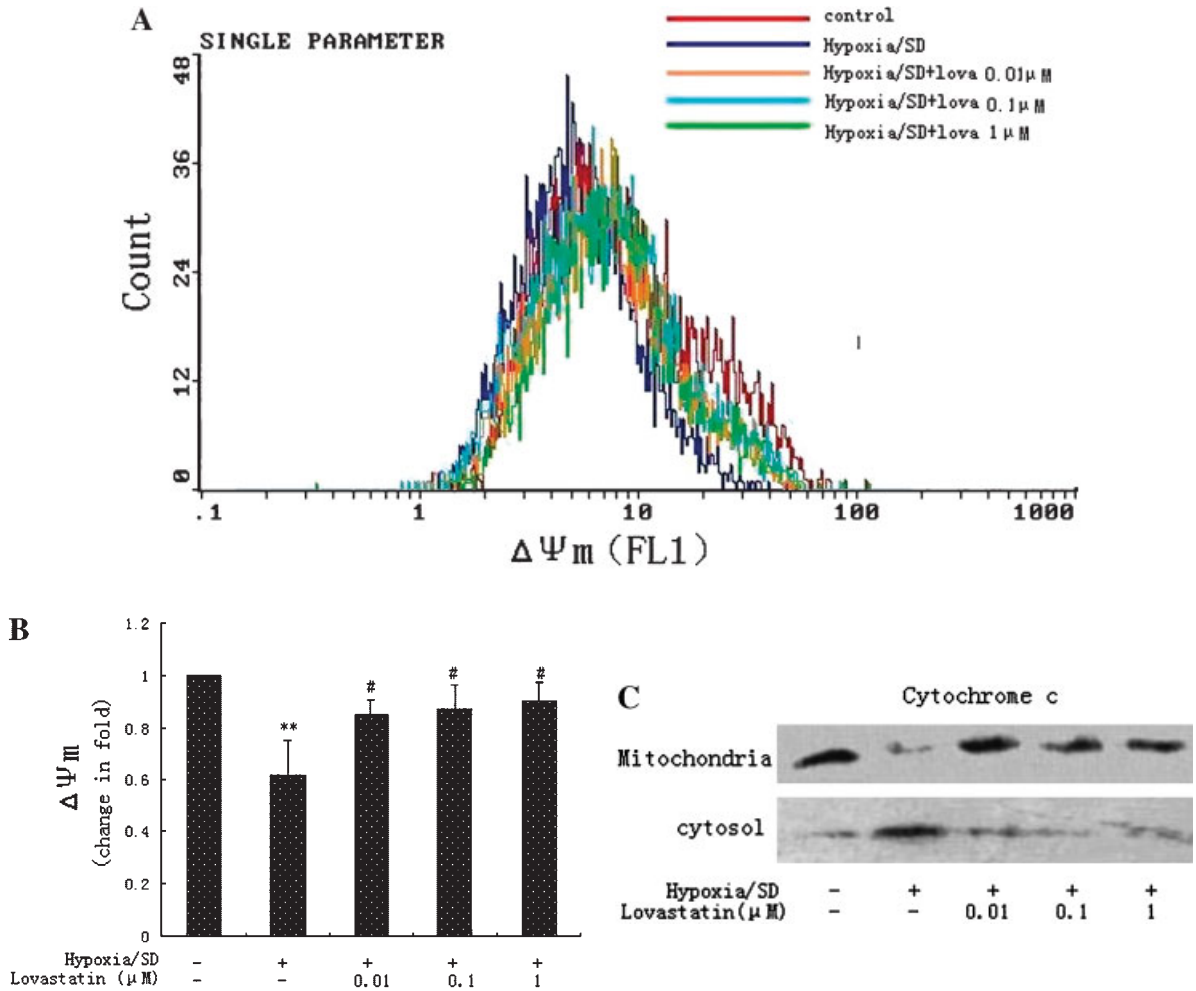

Fig. 3. Lovastatin inhibits Hypoxia/SD-induced loss of mitochondrial membrane potential (△Ψm) and cytochrome-*c* release. (**A**, **B**): Lovastatin inhibits loss of △Ψm detected by flow cytometry of Rhodanmine 123-stained cells. **C**: Western blot of cytosolic and mitochondrial fractions of MSCs shows a clear reduction of cytochrome-*c* release with lovastatin treatment before Hypoxia/SD. Each data point represents mean ± standard deviation of three independent experiments. $^{**}P < 0.01$ compared with control cells. $^{\#}P < 0.05$ compared with Hypoxia/SD cells.

increased at 5 min, but peaked at 40 min (Fig. 5A). Pretreatment of cells with LY294002 (25 μM) for 60 min potently inhibited PI3K activity induced by lovastatin (Fig. 5B), indicating that Akt reacted at the downstream of PI3K in lovastatin-mediated antiapoptotic pathway.

Akt is one of the critical regulators of GSK3β. Activated Akt phosphorylates Ser-9 of GSK3β, which inhibits GSK3β activity, and phosphorylation and inactivation of GSK3β may mediate some of the antiapoptotic effects of Akt [Cai et al., 2006]. To determine whether GSK3β was phosphorylated by activated Akt after lovastatin stimulation, we performed Western blot assays using anti-phospho-GSK3β antibody. Figure 5C showed a time-related phosphorylation of GSK3β when MSCs were treated with lovastatin, and interestingly, the PI3K inhibitor LY294002 completely suppressed the phosphorylation of GSK3β induced by lovastatin. This suggests that lovastatin stimulated cell survival through the PI3K/Akt/GSK3β signaling pathway.

Lovastatin Also Induced Specific MEK/ERK1/2 Phosphorylation

The mitogen-activated protein kinase (MAPK) pathway is another major cell survival mechanism. Treatment of MSCs with lovastatin

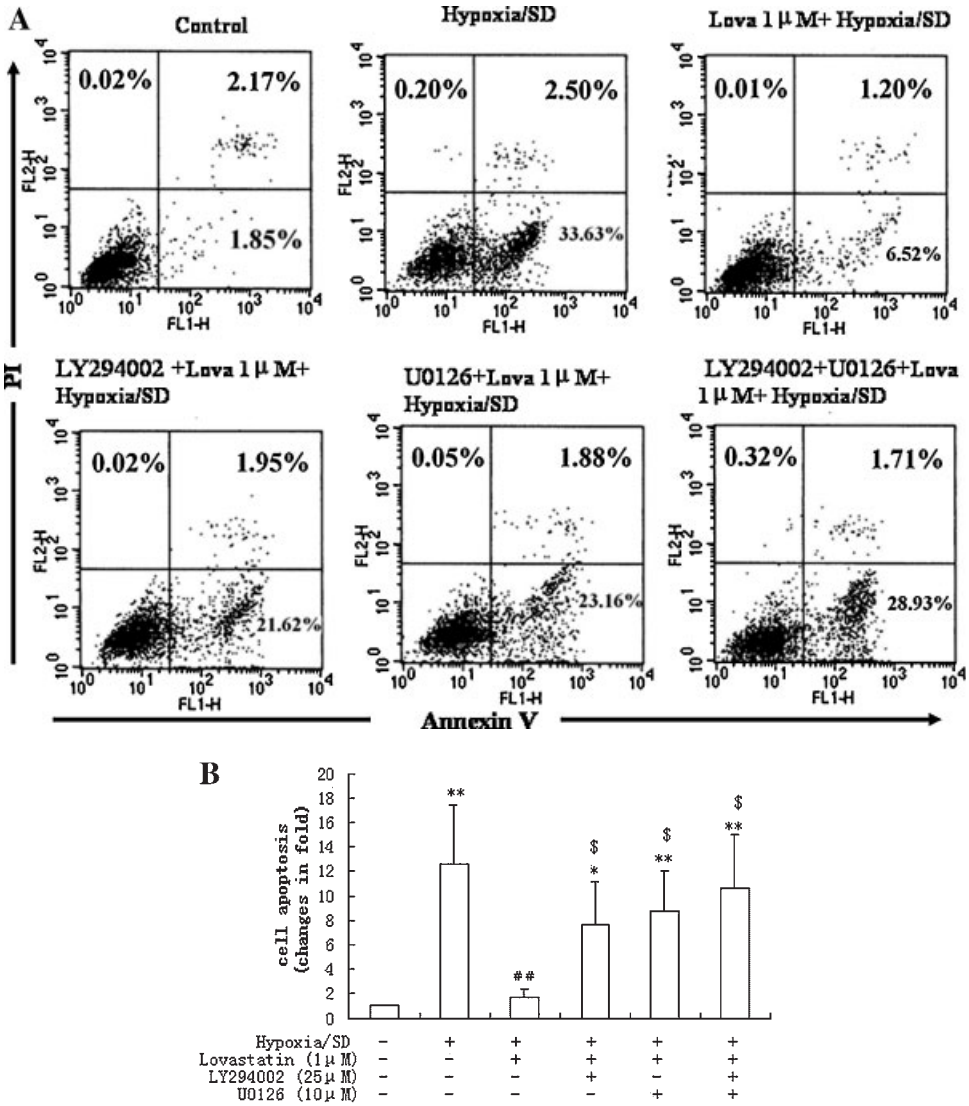

Fig. 4. Lovastatin prevents MSCs from Hypoxia/SD-induced through PI3K/Akt and ERK1/2 Pathways. **A**, **B**: MSCs were pretreated with or without a PI3K inhibitor, LY294002 (25 μM) and/or a MEK/ERK1/2 inhibitor, U0126 (10 μM) for 1 h followed by lovastatin incubation during Hypoxia/SD and then FACS analysis after staining with Annexin V and PI showed LY294002 significantly increased the population of early apoptotic cells (Annexin V+/PI−). Each data point represents mean ± standard deviation of three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.01$ compared with control cells. $^{\#\#}P < 0.01$ compared with Hypoxia/SD cells. $^{\$}P < 0.05$ compared with lovastatin and Hypoxia/SD.

protected MSCs against Hypoxia/SD-induced apoptosis. Flow cytometric analysis showed that the antiapoptotic effect of lovastatin was potently decreased when pretreating cells with an ERK1/2 inhibitor U0126, and the population of early apoptotic cells significantly increased ($P < 0.01$) (Fig. 4A,B).

We subsequently investigated whether lovastatin stimulated intracellular ERK1/2 phosphorylation events. After depletion of serum for 12 h, MSCs were stimulated with lovastatin (1 μM) and cells were harvested at 0, 5, 10, 20, 30, 40, 60, and 120 min respectively. Immunoblotting was conducted using anti-phospho-ERK1/2 antibody. Figure 6A showed that activation of ERK1/2 was observed within 20 min of initiating treatment, was sustained at a high level until 60 min, and returned to baseline by 2 h post-treatment. The activation of ERK1/2 was also blocked by U0126 (10 μM) (Fig. 6B).

In the present study we further investigated the potential interactions between these pathways and the results showed that activation of

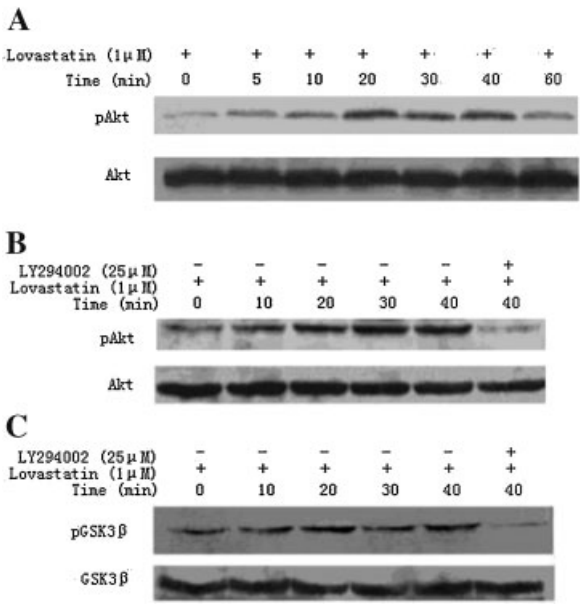

Fig. 5. Lovastatin phosphorylates Akt and GSK3β. After serum-starvation for (**A**) MSCs were stimulated with lovastatin (1 μM) for the indicated times and then the expressions of Akt and Phospho-Akt (Ser473) was analyzed by Western blot. **B**: Pretreatment of cells with LY294002 (25 μM) for 1 h potently inhibited Phospho-Akt activity induced by lovastatin. **C**: A time-related phosphorylation of GSK3β stimulated by lovastatin were completely suppressed by LY294002.

ERK1/2 was inhibited by the PI3K inhibitor LY294002 (Fig. 7A), but inhibition of ERK1/2 by U0126 did not inhibit phosphorylation of Akt and GSK3β (Fig. 7B, C).

LY294002 and/or U0126 Blocked the Inhibition of Cytochrome-*c* Release and Caspase-3 Activation by Lovastatin

MSCs were pretreated for 1 h with a PI3K inhibitor LY294002 (25 μM) or an ERK1/2 inhibitor U0126 (10 μM) before the addition of lovastatin, and then harvested for Western blot using anti-cytochrome-*c* antibody and anti-pro-caspase-3 antibody. The results indicated significant cytochrome-*c* release (Fig. 8A) and augmentation of the 19-kDa active form of caspase-3 (Fig. 8B). Further experiments revealed that LY294002 or U0126 abolished the inhibition of caspase-3 activity induced by lovastatin, and the combination of LY294002 and U0126 abrogated the inhibition of caspase-3 activity by lovastatin (Fig. 8C).

DISCUSSION

The major finding from this study was that lovastatin has a significant antiapoptotic effect on MSCs subjected to hypoxia and serum deprivation. The mechanism of this action was mediated through a mitochondrial pathway, inhibiting cytochrome-*c* release and reducing caspase-3 activation. We further showed that the protective mechanisms involved activation of PI3K/Akt and MEK/ERK1/2 cell survival signaling, revealing these as potential target pathways for antiapoptosis therapies in MSCs.

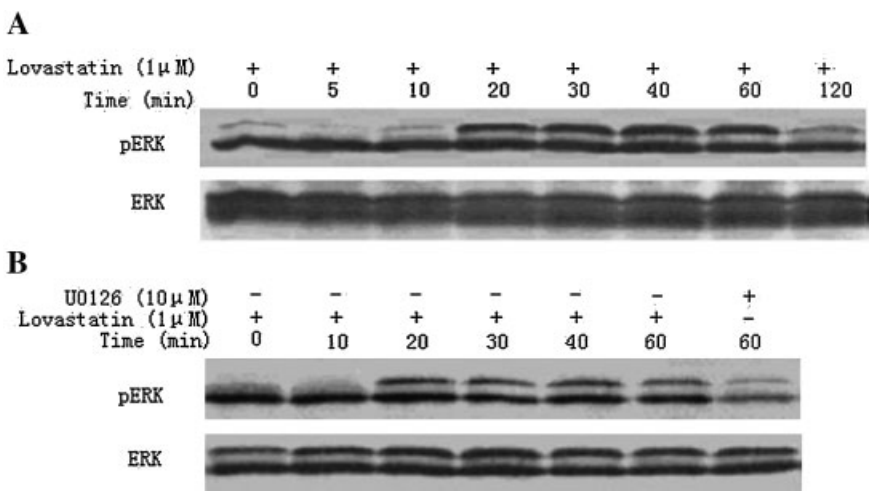

Fig. 6. Lovastatin phosphorylates ERK1/2 and U0126 inhibits the phosphorylation induced by lovastatin. MSCs were stimulated with lovastatin (1 μM) after serum-starvation for 12 h and cells were harvested at 0, 5, 10, 20, 30, 40, 60, and 120 min respectively. Western blot by using anti-p44/42 MAP kinase and anti-Phospho-p44/42 MAP kinase (Thr202/Tyr204) showed (**A**) phosphorylation of ERK1/2 (pERK1/2) was time-dependent. **B**: Pretreatment of cells with U0126 (10 μM) for 1 h potently inhibited ERK1/2 activity induced by lovastatin.

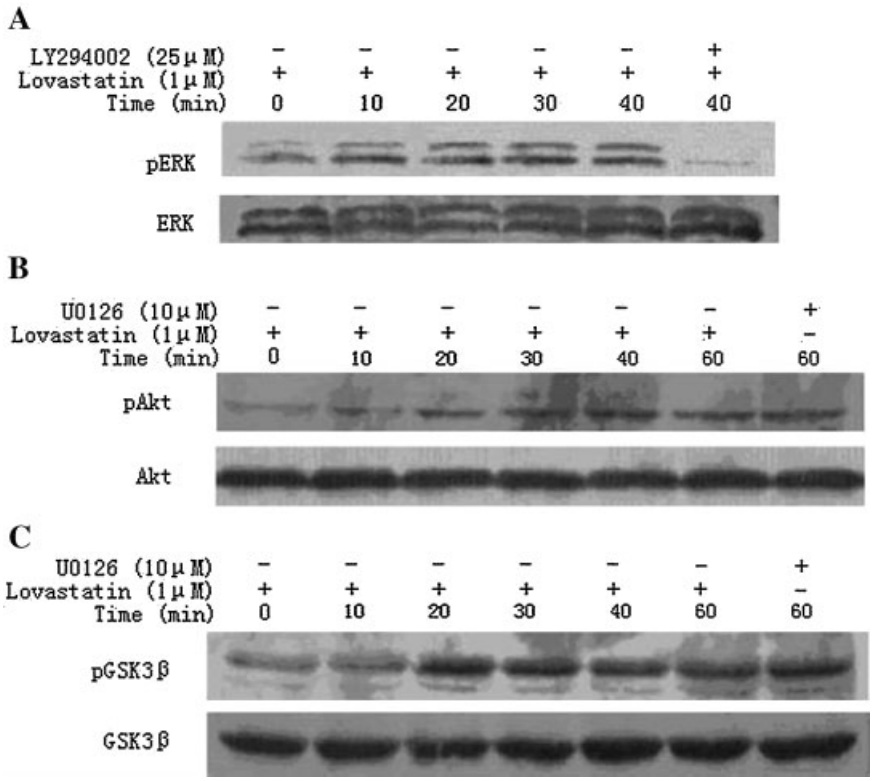

Fig. 7. The potential interactions between PI3K/Akt and MEK/ERK1/2 pathways activated by lovastatin. MSCs were stimulated with lovastatin (1 μM) after serum-starvation for 12 h and then harvested at 0, 5, 10, 20, 30, 40, and 60 min respectively. Western blot showed (**A**) pretreatment of cells with the PI3K inhibitor, LY294002 (25 μM) for 1 h potently inhibited ERK1/2 activity induced by lovastatin. **B**, **C**: Showed phosphorylation of Akt and GSK3β was not changed by the ERK1/2 inhibitor, U0126.

We previously reported that the early apoptosis in MSCs induced by Hypoxia/SD peaked at 6 h [Zhu et al., 2006]. Using the same model, we now show for the first time that lovastatin protected MSCs from apoptosis, and this effect was evident over a broad range of concentrations (0.01–1 μM). Interestingly, lovastatin inhibited only the early apoptosis mechanisms of MSCs induced by Hypoxia/SD but had no effect on late apoptosis or necrosis mechanisms. That this timing may be consistent with early mitochondrial pathways being important in this mechanism was confirmed by lovastatin significantly inhibiting caspase-3 activation and the presence of activated caspase-3 fragments in response to Hypoxia/SD.

Although several studies have suggested that statins may induce apoptosis in different cell lines [Cerezo-Guisado et al., 2005; Ajith et al., 2006], our data showed that under our experimental conditions (i.e., at low concentration and after short time of exposure) lovastatin did not induce apoptotic death, but exerted protective effects against Hypoxia/SD-induced apoptosis in MSCs. These are agreement with the studies by Renate et al. [Bardeleben et al., 2003] and Nubel et al. [2006] indicating that lovastatin inhibits apoptosis induced by ultraviolet light or ionizing radiation at low concentrations. Besides considering concentration, whether or not statins initiate apoptosis or whether they exert opposite effects, that is, protecting cells from Hypoxia/SD-induced cell death may be mostly cell type-dependent.

The present experiment data showed that the loss of $\triangle\Psi m$ and cytochrome-*c* release induced by Hypoxia/SD were both inhibited by lovastatin treatment at concentrations of 0.01–1 μM. These results were consistent with several earlier studies showing that preservation of mitochondrial function contributed to preventing apoptosis [Tiziano et al., 2004; Nishikawa et al., 2006]. Mitochondria are not only crucial for energy production but also play a critical role in apoptosis. Cytochrome-*c* resides primarily in the intermembrane space of the mitochondria

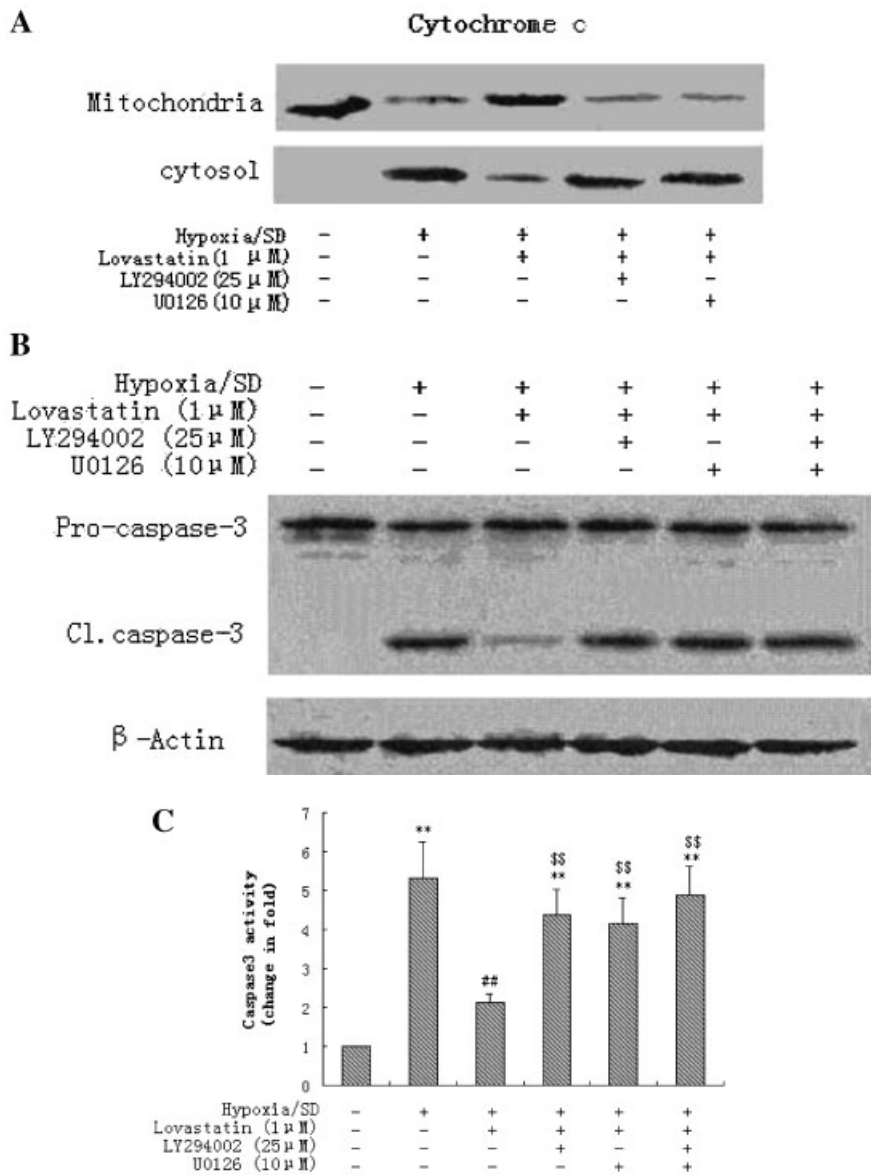

Fig. 8. Lovasatin inhibits Hypoxia/SD-induced cytochrome-*c* release and caspase-3 activation through PI3K and ERK signaling pathways. MSCs were pretreated with or without LY294002 (25 μM) or/and U0126 (10 μM) for 1 h followed by lovastatin incubation during Hypoxia/SD. **A**, **B**: Western blot analysis showed a clear increase of cytochrome-*c* release and active form of caspase-3 (Cl. Caspase-3) induced by LY294002 or/and U0126. **C**: LY294002 and U0126 abrogated the inhibition of caspase-3 activity by lovastatin in Hypoxia/SD-induced MSCs apoptosis. Each data point represents mean ± standard deviation of three independent experiments. $^{**}P < 0.01$ compared with control cells. $^{\#\#}P < 0.01$ compared with Hypoxia/SD cells. $^{\$\$}P < 0.01$ compared with lovastatin and Hypoxia/SD.

and is released into the cytosol in response to various apoptotic agents [Mano et al., 2004]. Our previous study found that Hypoxia/SD induced the loss of mitochondrial membrane potential and cytochrome-*c* release from the mitochondria to the cytosol in MSCs. Several other studies have shown that loss of $\triangle\Psi m$ and cytochrome-*c* release during the early phase of apoptosis [Korsnes et al., 2006] and attenuation of early phase cell apoptosis by enhancing mitochondrial function resulted in a significant prevention of the development of diabetic cardiomyopathy [Cai et al., 2006], suggesting more potential widespread applications for lovastatin therapy in the heart.

In this study, we investigated the role of PI3K/Akt pathway in MSCS survival to determine its role in the antiapoptotic effect of lovastatin. PI3k/Akt and MEK/ERK1/2 are important cell survival pathways, and

phosphorylation of Akt and ERK1/2 mediate antiapoptosis in a variety of cells [Uchiyama et al., 2004; Leeds et al., 2005; Kwon et al., 2006]. Activation of the Akt pathway results in phosphorylation and inactivation of the proapoptotic protein GSK3β, thus preventing apoptosis [Kim et al., 2003; Wong et al., 2005]. Statins increase survival of isolated human pancreatic islets by activating Akt [Contreras et al., 2002] and inhibit cardiomyocyte apoptosis through GSK3β inactivation [Bergmann et al., 2004]. Previous reports also showed that MSCS transduced with Akt had increased survival after transplantation into the ischemic porcine heart [Lim et al., 2006]. In support of the role of this pathway, we found that the PI3K inhibitor LY294002 attenuated lovastatin-mediated antiapoptosis, suggesting an important role for Akt in protection of MSCS from apoptosis induced by Hypoxia/SD. Further evidence for Akt in lovastatin-mediated antiapoptosis is phosphorylation of Akt in a PI3K-dependent manner. Following Akt phosphorylation, the phosphorylation and inactivation of GSK3β was also detected and inhibited by LY294002. Collectively, these data strongly argue for a crucial role of PI3K/Akt and its downstream target GSK3β in mediating the anti-apoptotic effect of lovastatin.

In a similar manner to PI3K inhibition, blockade of the MEK/ERK1/2 signaling pathway with an ERK1/2 inhibitor, U0126, also prevented the protective effects of lovastatin in Hypoxia/SD-induced MSCs apoptosis. Further results showed that the effect of lovastatin on ERK1/2 phosphorylation was prevented by U0126. Together, these studies implicated both the PI3K/Akt and MEK/ERK1/2 signaling pathways involved in the antiapoptotic effects of lovastatin.

In our study, whereas inhibiting PI3K or MEK significantly inhibited lovastatin's antiapoptotic effect on MSCs, simultaneous inhibition of both PI3K and MEK abolished the protective effect of lovastatin, indicating that some synergism between PI3K and MEK signaling exists. This phenomenon is in agreement with the study by Xie et al. [2005] showing that combined use of inhibitors of PI3K and MEK results in more severe apoptosis of immature monocyte-derived dendritic cells than that by a single inhibitor.

The potential interactions between these pathways were investigated in the present study, and interestingly the results showed that activation of ERK1/2 was inhibited by the PI3K inhibitor LY294002 but inhibition of ERK1/2 by U0126 did not inhibit phosphorylation of Akt and GSK3β, which implied that some proteins downstream of PI3K likely activate ERK signaling pathway. These results indicated that Akt and ERK1/2-mediated signaling might operate synergistically to reduce the apoptosis threshold in cells, in agreement with the study by Krishnan et al. [Dhandapani et al., 2005]. The emerging complex relationship between these pathways, as well as the potential role for statins in regulating the death receptor pathway of apoptosis [Chang et al., 2006] are areas worthy of further study in promoting MSCS survival under hypoxic/SD conditions.

Statins have been shown to ameliorate remodeling and to improve survival in animals after myocardial infarction and ischemia-reperfusion injury [Bauersachs et al., 2001; Hayashidani et al., 2002; Yasuda et al., 2007]. Clinical trails have suggested that statin therapy appears to reduce the incidence of stroke and myocardial infarction [Gröschel et al., 2006], and exert beneficial effects on the clinical course of myocardial failure in patients with or without coronary artery disease [Horwich et al., 2004]. These basic and clinical observations have led to the suggestion that statins may be of value in the prevention and treatment of patients with myocardial infarction and heart dysfunction [Maack et al., 2003; von Haehling et al., 2003]. Our findings suggest that a clinical application for this lovastatin-mediated antiapoptotic effect in MSCs would likely involve a statin pretreatment model for MSCs before cardiac cell therapy, to determine if the same mechanism may effectively increase transplanted cells survival.

Taken together, these data strongly indicate that lovastatin protects MSCs against Hypoxia/SD-induced apoptosis through the activation of the mitochondrial pathway, preventing cytochrome-*c* release and caspase-3/CPP32 activation. Furthermore both PI3K/Akt and MEK/ERK1/2 cell survival signaling pathways were involved in the anti-apoptosis mechanism mediated by lovastatin. This study provides a promising approach for promoting MSCS survival with a widely available and acceptable therapeutic agent. And our next study would aim to investigate the influence of statins on

MSC survival in vivo. It may have considerable significance in improving the efficiency of stem cell therapy after myocardial injury.

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