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Hypoxic preconditioning attenuates hypoxia/reoxygenation-induced apoptosis in mesenchymal stem cells[1]

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Key words

mesenchymal stem cells; apoptosis; hypoxia preconditioning; mitochondrial transmembrane potential; Bcl-2; extracellular regulated kinase1/2; Akt; vascular endothelial growth factor

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Abstract

Aim: Mesenchymal stem cells (MSC) are a promising candidate for cardiac replacement therapies. However, the majority of transplanted MSC are readily lost after transplantation because of poor blood supply, ischemia-reperfusion, and inflammatory factors. We aimed to study the effects of hypoxia preconditioning (HPC) on hypoxia/reoxygenation-induced apoptosis of MSC. **Methods:** Three generations of MSC were divided into 6 groups, including the normal group, hypoxia-reoxygenation (H/R) group, cyclosporine A (CsA), and the HPC 10 min, 20 min, and 30 min groups. The apoptotic index, cell viability, mitochondrial membrane potential, translocation of Bcl-2 and bax, extracellular regulated kinase (ERK), Akt, hypoxia-inducible factor 1-α, and the vascular endothelial growth factor (VEGF) were tested after H/R treatment. **Results:** HPC decreased the apoptotic index and increased the viability induced by H/R. Moreover, HPC markedly stabilized mitochondrial membrane potential, upregulated Bcl-2 and VEGF expressions, and increased the phosphorylation of ERK and Akt. As a positive control, CsA has the same function as HPC, except for promoting ERK and Akt phosphorylation and upregulating VEGF. **Conclusion:** HPC had a protective effect against MSC apoptosis induced by H/R via stabilizing mitochondrial membrane potential, upregulating Bcl-2 and VEGF, and promoting ERK and Akt phosphorylation. HPC has implications for the development of novel stem cell protective strategies.

Introduction

Although there are many therapeutic advances in myocardial infarction, the irreversible loss of cardiomyocytes remains a key problem to resolve, and this forms the cellular basis of cardiac dysfunction. Mesenchymal stem cell (MSC) transplantation is a promising strategy. MSC can differentiate into vascular endothelial cells and cardiomyocytes as well as improve heart function[1,2]. However, this cell replacement therapy is limited by their poor viability after transplantation. Approximately 99% of transplanted MSC are readily lost during the first 24 h after transplantation[3]. Apoptosis is thought to be a major factor in their demise. Therefore, protecting MSC against apoptosis in a pro-apoptotic microenvironment of the infarcted heart is critical for improving the efficiency of cell therapy. Hypoxia preconditioning (HPC) increases cellular resistance against subsequent lethal hypoxia injury. It has been documented in various preparations, including cultured myocytes[4]. However, the effect of HPC on the apoptosis in MSC is still unclear. Thus, we aimed to study the protective effect of HPC on MSC against hypoxia/reoxygenation (H/R)-induced injuries. The effects of HPC on mitochondrial transmembrane potential ($\Delta\Psi m$), extracellular regulated kinase (ERK)-1/2, Akt, hypoxia-inducible factor 1 (HIF-1)α, the vascular endothelial growth factor (VEGF), and the translocation of Bcl-2 and bax were also examined.

In the present study, we verified our hypothesis that

HPC attenuates apoptosis in isolated MSC induced by H/R, which imitated myocardial ischemic–reperfusion.

Materials and methods

Isolation and proliferation of MSC The isolation and proliferation of MSC was performed according to previously described methods[5]. In brief, we humanely killed male Sprague–Dawley rats (80 g) and harvested the bone marrow by flushing their femoral and tibial cavities with phosphate-buffered saline (PBS). Bone marrow cells were prepared by gradient centrifugation at 900×g for 30 min on a Percoll gradient (GE Healthcare, Sweden) at a density of 1.073 g/mL and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum. A small number of cells developed visible symmetric colonies from d 5 to 7. Non-adherent hematopoietic cells were removed by medium changes. The adherent, spindle-shaped MSC population expanded to $>5\times10^7$ cells within 3 passages. Cells were determined by fluorescence-activating cell sorting analysis (Beckman Coulter, Fullerton, CA USA) before this experiment using directly conjugated antibodies against anti-rat CD44, anti-CD45, and anti-CD90 (PE, Caltag, Burlingame, CA USA). The study was approved by the ethics committee of Zhejiang University (Hangzhou, China).

Experimental protocols All of the cells in the study were at passage 3. Cultured MSC were divided into six groups: (i) group 1: normal MSC. Normal MSC were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics during the entire experimental period; (ii) group 2: H/R. MSC were cultured in DMEM without fetal bovine serum and exposed to hypoxia for 6 h (<0.5% O_2) in an airtight chamber (Billups–Rothenberg, Del Mar, CA, USA), followed by 12 h of reoxygenation (21% O_2). The oxygen level in the chamber was monitored with an oxygen analyzer; (iii) group 3: cyclosporine A (CsA)+H/R. As a positive control group, MSC were pre-incubated with 0.5 μmol/L CsA (Sigma–Aldrich, St Louis, MO, USA) for 30 min before H/R. CsA is a strong inhibitor of mitochondrial permeability transition pore and thus reduces cell apoptosis; (iv) group 4: HPC 10 min+H/R. HPC was induced by incubating the MSC in an airtight chamber in which air was replaced by 8% O_2. HPC was induced by exposing the MSC to 10 min of hypoxia and 30 min of reoxygenation before H/R treatment; (v) group 5: HPC 20 min+H/R. HPC was induced by exposing the MSC to 20 min of hypoxia and 30 min of reoxygenation before H/R treatment; and (vi) group 6: HPC 30 min+H/R. HPC was induced by exposing the MSC to 30 min of hypoxia and 30 min of reoxygenation before H/R treatment.

Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling assay Terminal deoxynucleotidyl transferase-mediated dUTP-nick-end labeling (TUNEL; Roche Diagnostics, Mannheim Germany) was performed with a detection kit according to the manufacturer's instructions. Briefly, the cells were fixed with 4% paraformaldehyde at room temperature for 60 min and subsequently permeabilized with 0.1% Triton X 100 for 30 min. After the cells were incubated with TUNEL reaction mixture for 60 min at 37 °C in a humidified chamber, the cells was incubated in converter-peroxidase (POD) for 30 min at 37 °C, and then incubated with a diaminobenzidine POD substrate .The percentage of TUNEL-positive cells in the total number of cells was determined by counting at least 200 cells in 3 different fields. The experiment was performed 3 times.

Cell viability assay The MSC were seeded in 96-well plates at a density of 5×10^4 cells/well. After H/R treatment, the cells were incubated with 500 μg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich, USA) and cultured for 3 h in a CO_2 incubator. Functional mitochondrial succinate dehydrogenase in cells can convert MTT to formazan that generates a blue color when dissolved in dimethylsulfoxide. After the purple formazan producer was dissolved, the intensity was measured with a microplate reader at an absorption wavelength of 570 nm.

ΔΨm ΔΨm was assessed using the lipophilic cationic probe JC-1 (BioVision, Mountain View, CAUSA), a sensitive fluorescent dye. The red emission of the dye is attributable to a potentially-dependent aggregation in the mitochondria, reflecting ΔΨm. Green fluorescence reflects the monomeric form of 5, 5', 6, 6' -tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide -1 (JC-1), appearing in the cytosol after mitochondrial membrane potential depolarization. The MSC were loaded with 10 μmol/L JC-1 for 15 min and 5 μg/mL Hoechst 33342 (Sigma-Aldrich, USA) for 0.5 min at 37 °C, respectively. The fluorescence was monitored by the Zeiss fluorescence microscopy system. The percentage of the loss of ΔΨm cells in the total number of cells was determined by counting at least 200 cells in 3 different fields.

Isolation of mitochondria and cytosol Mitochondrial and cytosolic fractions were isolated using a mitochondria/cytosol fractionation kit (BioVision, USA) according to the manufacturer's protocol. Briefly, the cells were collected by centrifugation at 600×*g* for 5 min at 4 °C. The cells were then resuspended in 1 mL of 1× cytosol extraction buffer, incubated on ice for 10 min, and homogenized in an ice-cold tissue grinder for 60 passes. The cell homogenate was sub-

jected to 700×*g* centrifugation for 10 min. The resultant supernatant was further centrifuged at 10 000×*g* for 30 min. The supernatants were used as the cytosolic fraction, while the pellets were then resuspended in 100 μL of the mitochondrial extraction buffer to isolate the mitochondria.

Western blot analysis For the detection of the protein expression, the disposed cells were washed with PBS and then scraped into 50 μL lysis buffer consisting of 50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1% Triton X-100, 0.5 % Nonidet P NP-40, 1 mmol/L EDTA, 1 mmol/L *O*,*O*′-Bis ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L sodium fluoride, and 5 mg/mL aprotinin. After being quantitated by bicinchoninic acid reagent, equivalent proteins for each sample were resuspended in 10 μL electrophoresis sample buffer (10 mmol/L Tris-HCl, pH 6.8, 100 mmol/L dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol), boiled for 5 min, and subjected to SDS–PAGE in 10%–15% acrylamide mini-gels. The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane for 2 h in a transfer buffer (25 mmol/L Tris, 200 mmol/L glycine, and 20% methanol). After being blocked with 5% skim milk in Tris-buffered saline (TBS) for 1 h at room temperature, the PVDF membrane was washed 3 times in TBS containing 0.1% Tween-20 and incubated with a protein-specific antibody for 12 h at room temperature followed by a secondary antibody. Primary antibodies were used as follows: antibodies specific to Bcl-2, bax, Akt, and phospho-Akt (Cell Signaling Technology, Beverly, MA USA), an antibody specific to HIF-1α (R&D Systems, Minneapolis, MN USA), antibodies specific to VEGF, β-actin, ERK, and phosphor-ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with the secondary antibody, the membranes were rinsed and the horseradish peroxidase labels were detected by enhanced chemiluminescence (Santa Cruz Biotechnology, USA) followed by autoradiography. Image Pro Plus 5 software(Media Cybernetics, Georgia, MD, USA) was used to semiquantify the protein level in every lane.

Statistical analysis The statistical analysis was performed with one-way ANOVA followed by a Bonferroni multiple-comparison test from more than 3 independent experiments. The results were considered significant at a value of $P<0.05$.

Results

Surface analysis of MSC line by flow cytometry The surface markers for MSC were identified. The putative MSC used in the experiments expressed CD44 and CD90 at moderate to high levels (86.5% and 99.4%, respectively), but CD45 was at a low level (4.47%; Figure 1).

HPC prevented MSC from H/R-induced apoptosis and increased cell viability We first examined whether HPC prevented the MSC from H/R-induced apoptosis. In our experimental conditions, approximately 3% of normal MSC of the 3 generations were TUNEL-positive, as reported earlier[6] (Figure 2A). H/R markedly increased TUNEL-positive MSC compared with normal cells (48.2%±3.1%, $P<0.05$ *vs* normal cells; Figure 2). However, when the MSC were pretreated by HPC for 10, 20, and 30 min before H/R, the number of TUNEL-positive MSC significantly decreased in a time-dependent manner (HPC 10 min 20.4%±1.3%, HPC 20 min 16.8%±1.5%, and HPC 30 min 14.2%±0.5%, $P<0.05$ *vs* H/R; Figure 2). HPC also increased the viability of the MSC in a time-dependent manner in response to H/R ($P<0.05$ *vs* H/R; Figure 2). At the same time, pre-incubation with 0.5 μmol/L CsA reduced TUNEL-positive MSC (22.0%±1.6%, $P<0.05$ *vs* H/R) and increased cell viability. To further investigate whether HPC has long-term beneficial effects on MSC, we tested the apoptosis induced by 24 h hypoxia and reoxygenation on the MSC. HPC 10 min and 30 min reduced the apoptotic index from 83%±5.6% to 56%±3.5%, and 42%±4.1% ($P<0.05$ *vs* H/R;

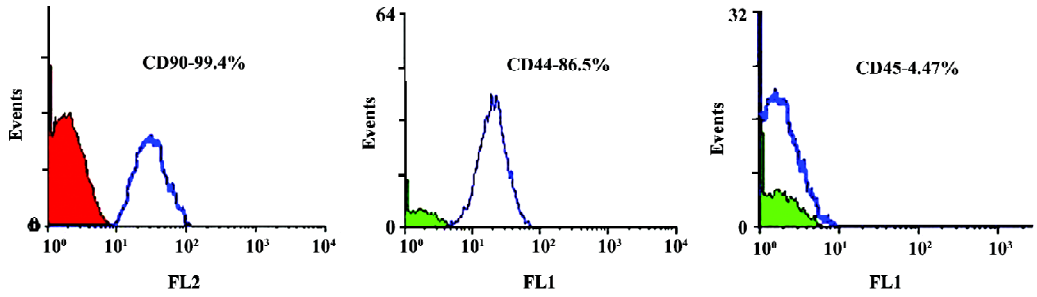

Figure 1. FACS analysis of MSCs with antibodies directed against CD44, CD90 and CD45 . X axis represents fluorescence intensity, while Y axis expressed cells counting.

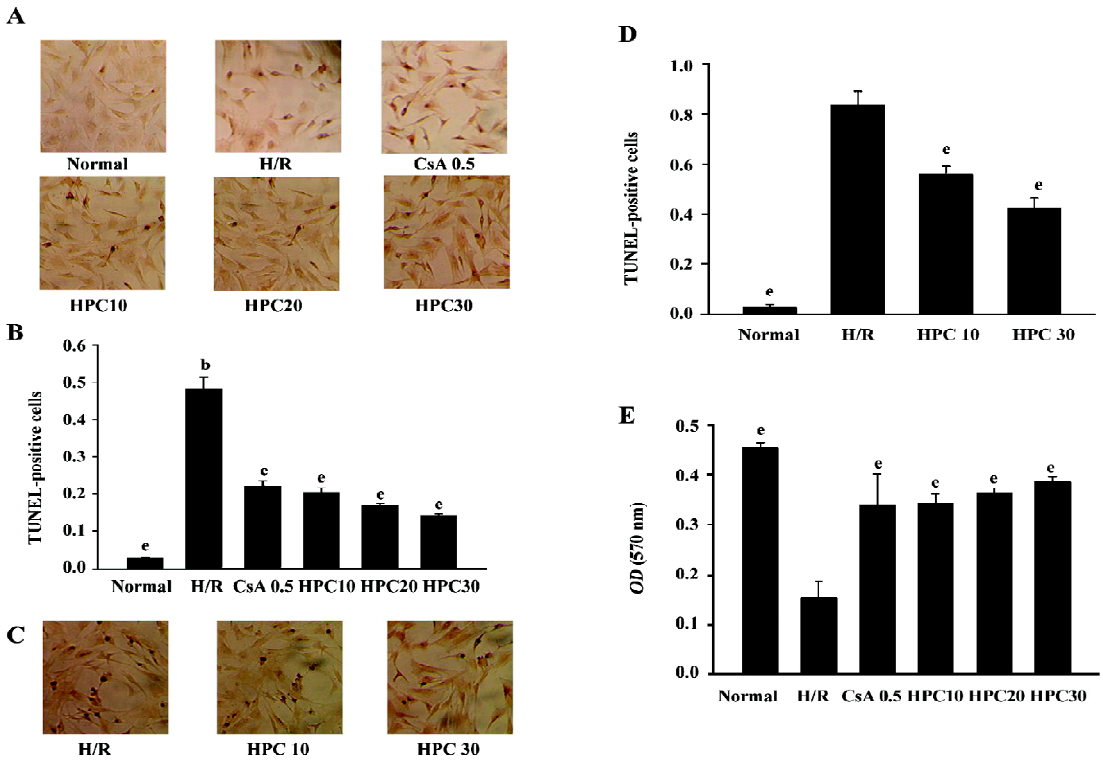

Figure 2. HPC inhibits H/R-induced apoptosis of MSCs and increased cell viability. (A) DNA strand breaks assessed by TUNEL staining of MSCs. Shown are photograph of MSCs of normal cell control; H/R (6 h hypoxia and 12 h reoxygenation); H/R and 0.5 μmol/L CsA; H/R and HPC 10 min; H/R and HPC 20 min; H/R and HPC 30 min. Brown nucleus indicates TUNEL-positive (apoptotic) cell. magnification×100. (B) Quantification of apoptotic MSCs by TUNEL staining. (C) Shown are photograph of apoptosis of MSCs of H/R(24 h hypoxia and 24 h reoxygenation); H/R and HPC10 min; H/R and HPC30 min.(D) Quantification of apoptotic MSCs by TUNEL staining. (E) Quantitative analysis of cell viability by MTT. Data shown are means±SEM representative of 3 independent experiments. $^{b}P<0.05$ *vs* normal cells; $^{e}P<0.05$ *vs* H/R group.

Figure 2C,2D).

Influence of HPC on H/R-induced ΔΨm To determine whether HPC influenced H/R-induced MSC mitochondrial dysfunction, we assessed ΔΨm using the potential-sensitive fluorescent probe JC-1. Normal MSC exhibited punctate red staining indicative of coupled mitochondria with a normal ΔΨm (Figure 3A). The MSC in the H/R group developed a diffuse green staining pattern, representative of reduced ΔΨm (Figure 3). HPC and CsA had a marked effect on JC-1 staining, which revealed HPC and CsA could preserve ΔΨm (Figure 3A,3B).

Effect of HPC on the translocation of Bcl-2 and bax in mitochondria To investigate whether HPC affected apoptosis-regulating proteins induced by H/R, the MSC expressions of Bcl-2 and bax were determined by Western blotting. We first examined the expression of anti-apoptotic Bcl-2 proteins in the mitochondrial fraction of MSC that were exposed to H/R. The results showed that Bcl-2 levels were upregulated after H/R treatment by 2.3±0.18-fold versus normal cells ($P<0.05$), but HPC significantly increased the expression of the protein Bcl-2 compared with the H/R group (HPC 10 min 3.3±0.11, HPC 20 min 3.4±0.23, and HPC 30 min 3.8±0.11-fold *vs* normal cells; $P<0.05$, HPC *vs* H/R; Figure 4A, 4B). Pre-incubation with 0.5 μmol/L CsA upregulated the Bcl-2 expression by 2.68±0.18-fold versus normal cells ($P<0.05$, CsA *vs* H/R). This suggested that the Bcl-2 upregulation induced by H/R could be enhanced by HPC treatment.

We next determined whether HPC had any effects on the levels of bax proteins in mitochondrial fraction (Figure 4A–4E). H/R did not induce bax translocation in our study (1.06±0.15-fold *vs* normal cells; $P>0.05$, H/R *vs* normal cells). However, HPC 10 min increased the levels of the pro-apoptotic protein bax by 1.45±0.17-fold versus normal cells ($P<0.05$, HPC 10 min *vs* H/R), HPC 20 and 30 min and CsA did not have any effect on bax translocation (1.45±0.17-fold *vs* normal cells, HPC 20 min 1.21± 0.18, HPC 30 min 1.07±0.14, and CsA 1.12±0.15-fold *vs* normal cells; $P>0.05$; Figure 4A–4E). At the same time, HPC and CsA upregulated Bcl-2 in the mitochondrial fraction and downregulated bax expression in the cytosol fraction ($P<0.05$, HPC *vs* H/R; Figure 4A–4E).

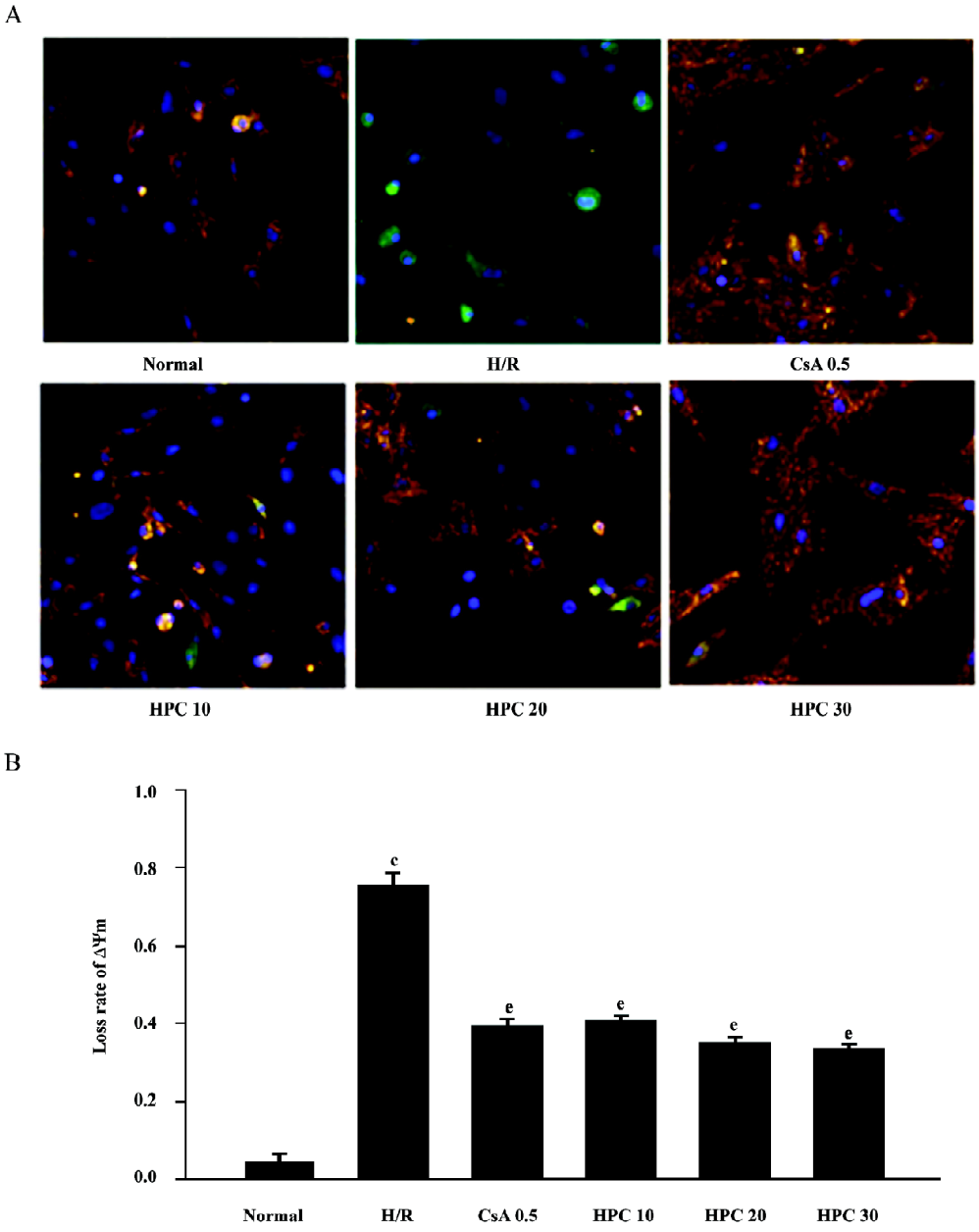

Figure 3. Influence of HPC on H/R-induced mitochondrial membrane potential of MSCs. (A) mitochondrial membrane potential (ΔΨm) of MSCs exposed to H/R in the absence or presence of HPC or 0.5 μmol/L CsA was determined using the potential-sensitive fluorescent probe JC-1. Meanwhile, DNA was stained with Hoechst 33342. magnification×200. Each panel shows an overlay of 3 images: orange-yellow color denotes hyperpolarized (aggregate) and green fluorescence signals indicates depolarized. Blue fluorescence indicates nuclei. (B) Quantification of loss rate of ΔΨm. Results are representative of 1 experiment from a total of 3 experiments performed. $^cP<0.01$ *vs* normal. $^eP<0.05$ *vs* H/R group.

Effects of HPC on the activation of ERK-1/2 induced by H/R in MSC To further determine the mechanism underlying the effect of HPC on MSC, we tested the ERK1/2 expression by Western blot analysis. The results showed that the phospho-ERK content decreased in the MSC maintained after H/R by 0.44±0.17-fold versus normal cells ($P<0.05$, H/R *vs* normal cells; Figure 4). However, these effects induced by H/R were reversed by HPC, and HPC could time-dependently upregulate phospho-ERK (HPC 10 min 0.80±0.16, HPC 20 min 0.91±0.03, and HPC 30 min 1.04±0.04-fold *vs* normal cells; $P<0.05$, HPC *vs* H/R). However, phospho-ERK in the CsA group was not higher than that of the H/R group ($P>0.05$).

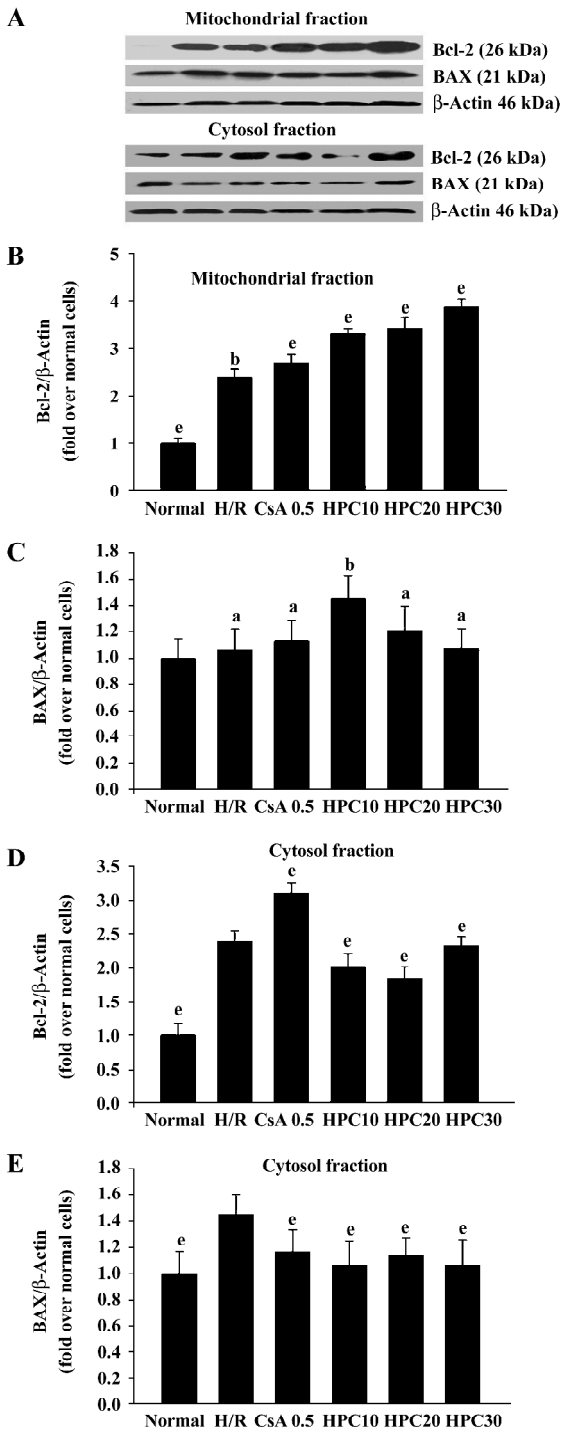

Figure 4. Effect of HPC on Bcl-2 and Bax translocation. MSCs were treated by HPC as indicated. (A)12 μg of the mitochondrial fraction and the cytosolic fraction was loaded onto an SDS-polyacrylamide gel, and Bcl-2 and Bax was detected by Western blotting.(B-E) Quantification of Bcl-2 and Bax expression in mitochondrial or cytosol fraction. Data shown are means±SEM. $^{a}P>0.05$, $^{b}P<0.05$ *vs* normal cells, $^{e}P<0.05$ *vs* H/R group. A representative blot of 3 independent experiments is shown.

HPC or CsA did not alter the ERK1/2 expression. These data suggested that ERK phosphorylation might be involved in the anti-apoptosis of HPC in the H/R-induced apoptosis of MSC.

Effects of HPC on the activation of Akt induced by H/R in MSC To further determine the underlying mechanism of the effect of HPC on MSC, we next tested the Akt expression, a molecule involved in the transduction of anti-apoptotic signals. The result showed that the phospho-Akt content decreased in the MSC after H/R treatment by 0.39±0.14-fold versus normal cells ($P<0.05$, H/R *vs* normal cells; Figure 6). However, these effects induced by H/R were reversed by HPC. HPC time-dependently upregulated the phospho-Akt level (HPC 10 min 0.43±0.13, HPC 20 min 0.47±0.12, and HPC 30 min 0.73±0.014-fold *vs* normal cells; $P<0.05$, HPC *vs* H/R). The level of phospho-Akt in the CsA group decreased ($P<0.05$, CsA *vs* H/R). HPC or CsA did not alter the Akt expression. This data suggested that Akt phosphorylation may be involved in the anti-apoptosis effect of HPC against H/R injury.

Effects of HPC on HIF-1α and VEGF expressions induced by H/R in MSC To further determine the underlying mechanism of the effect of HPC on MSC, we next tested the HIF-1α and VEGF expressions, the survival factors for some cell lines in response to hypoxia. After 6 h hypoxia and 12 h reoxygenation, the expression of HIF-1α did not significantly change in the MSC subjected to H/R or HPC (Figure 7A).

In our model, the VEGF content was increased in the

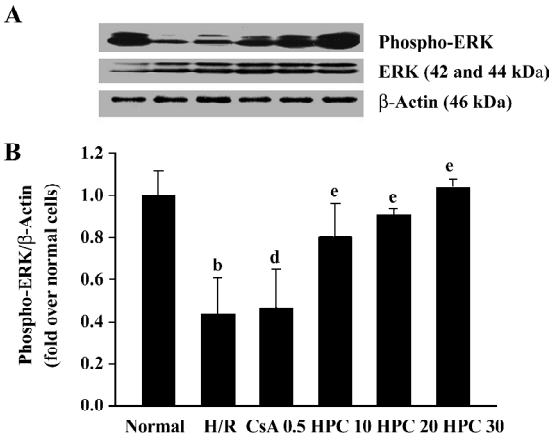

Figure 5. Effects of HPC on activation of ERK-1/2 induced by H/R in MSCs. (A)MSCs were pretreated by HPC before H/R stimulation. Activation of ERK-1/2 was determined by Western blot analysis, using antibody specific to activated ERK-1/2. (B) Quantification of phospho-ERK expression in normal cell control; H/R ; H/R and 0.5 μmol/L CsA; H/R and HPC 10 min; H/R and HPC 20 min ; H/R and HPC 30 min. Data shown are means±SEM. $^{b}P<0.05$ *vs* normal cells; $^{d}P>0.05$, $^{e}P<0.05$ *vs* H/R group. A representative blot of 3 independent experiments is shown.

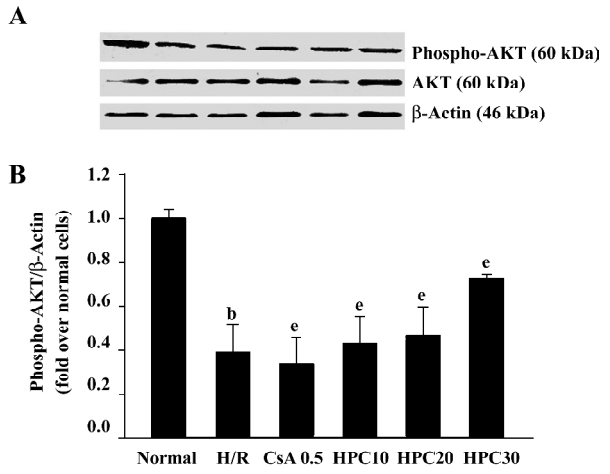

Figure 6. Effects of HPC on activation of Akt induced by H/R in MSCs. (A)MSCs were pretreated by HPC before H/R stimulation. Activation of Akt was determined by Western blot analysis, using antibody specific to activated Akt. (B) Quantification of phospho-Akt expression in normal cell control; H/R ; H/R and 0.5 μmol/L CsA; H/R and HPC 10 min; H/R and HPC 20 min; H/R and HPC 30 min. Data shown are means±SEM. $^{b}P<0.05$ *vs* normal cells; $^{e}P<0.05$ *vs* H/R group. A representative blot of 3 independent experiments is shown.

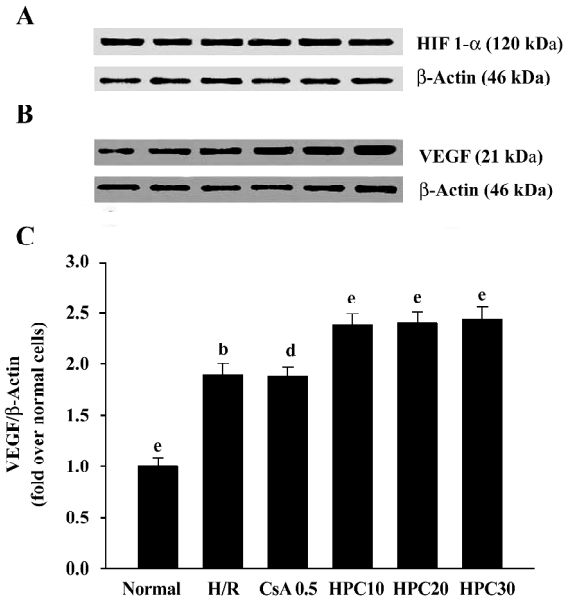

Figure 7. Effects of HPC on HIF 1-α and VEGF expression induced by H/R in MSCs. (A)MSCs were pretreated by HPC before H/R stimulation. HIF 1α was determined by Western blot analysis. (B) MSCs were pretreated by HPC before H/R stimulation. VEGF was determined by Western blot analysis.(C)Quantification of VEGF expression in normal cell; H/R; H/R and 0.5 μmol/L CsA; H/R and HPC 10 min; H/R and HPC 20 min; H/R and HPC 30 min. Data shown are means±SEM. $^{b}P<0.05$ *vs* normal cells; $^{d}P>0.05$ *vs* H/R group, $^{e}P<0.05$ *vs* H/R group. A representative blot of 3 independent experiments is shown.

MSC after H/R by 1.89±0.15-fold versus normal cells ($P<0.05$, HR *vs* normal cells). Meanwhile, these effects induced by H/R were enhanced by HPC. HPC time-dependently upregulated VEGF (HPC 10 min 2.37±0.12, HPC 20 min 2.40±0.10, and HPC 30 min 2.44±0.11-fold *vs* normal cells; $P<0.05$, HPC *vs* H/R). The VEGF expression in the CsA group had no difference compared with the H/R group ($P>0.05$, CsA *vs* H/R). This data suggested that VEGF may be involved in the effect of HPC on the H/R-induced apoptosis of MSC.

Discussion

Controlling the apoptosis of transplanted stem cells in a pro-apoptotic microenvironment has become a major challenge to stem cell therapy. Implanted MSC seem to be highly sensitive to ischemic environments[6]. Moreover, frequent ischemia attack will cause ischemic–reperfusion damage to implanted cells[7]. Several strategies were designed to inhibit the apoptosis of MSC after grafting. Heat-shock treatment[8] or a hypoxia-regulated Heme oxygenase-1 (HO-1) vector modification[9] of MSC could help cells to survive H/R injury. The transgenic expression of fibroblast growth factor-2[10] in MSC has been shown to improve the survival of the donor cell and cardiac function, suggesting that a major obstacle to successful tissue repair is the poor survival of transplanted stem cells. Some reports debate whether exogenous stem cells become new myocytes or secret cardioprotective proteins[11,12]. In either case, we expected that an improvement of grafted MSC survival will contribute to the refinement of cell transplantation efficiency.

The results of the present study demonstrate for the first time, at least in specific cell types, that HPC inhibits the H/R-induced apoptosis of MSC in a time-dependent manner by TUNEL assay. The protective effect of HPC was also confirmed by MTT, which was in agreement with the results of the TUNEL analysis. It should be noted, however, that HPC could not completely abolish all MSC death in our study.

The mechanism underlying the protective effect of HPC appeared to involve the stabilization of mitochondria, therefore reducing $\Delta\Psi m$ loss. HPC also increased anti-apoptotic protein Bcl-2 expression in mitochondria. The Bcl-2 protein played an important role in the regulation of mitochondrial dysfunction and apoptosis in some cell lines, which could stabilize mitochondrial permeability transition (MPT), thereby inhibiting the release of pro-apoptotic factors, such as apoptosis induce factor (AIF) and cytochrome c from the mitochondria[13].

This study demonstrated that the phosphorylation of Akt

and ERK1/2 markedly decreased in the H/R group. The downregulating effect of H/R on the levels of phospho-ERK1/2 and phospho-Akt was fully reversed by HPC. ERK1/2 belongs to the class of protein kinase signal transduction pathways that are used to relay numerous extracellular signals within cells and have been reported to be involved in various cellular functions, including apoptosis and proliferation[13]. We observed that H/R-induced apoptosis was at least mediated in part by the reduction of ERK1/2 phosphorylation in MSC, which was clearly inhibited by HPC. HPC enhanced ERK1/2 phosphorylation, which was believed to be one of the survival pathways triggered by H/R stress. These findings suggested that HPC protected the MSC against apoptosis via the ERK1/2 pathways. Akt, also referred to as protein kinase B or Rac, is thought to play a role in controlling the balance between survival and apoptosis by means of its ability to phosphorylate[15]. Akt confers survival signals, at least in part, through upregulating Bcl-2 expression[16]. Bcl-2 is also phosphorylated and this modification has been demonstrated to alter its anti-apoptotic activity[17]. In our study, HPC also stimulated the phosphorylation of Akt to reduce the apoptosis of MSC. MSC with Akt overexpression efficiently repaired infarcted rat myocardium and improved cardiac function[18].

Under low-oxygen conditions, cells develop an adaptive program that leads to the induction of several genes, which are transcriptionallyregulated by HIF-1. HIF-1 is a heterodimer consisting of α and β subunits. HIF-1α content is controlled by an intracellular O_2 concentration. HIF-1α content progressively increased as fractional inspired O_2 concentration FIO_2 fell below 21%. However, HIF-1α is rapidly degraded by O_2-dependent proline hydroxylation during reoxygenation[19]. The results of our experiment depicted in Figure 7 showed no difference in HIF-1α transcription in MSC after 6 h hypoxia and 12 h reoxygenation between normal cells, H/R, CsA, and the HPC groups, which could be explained by the degradation of HIF-1α.

VEGF is an angiogenic peptide that is released in response to hypoxia, which could be activated by HIF-1[20]. The angiogenic action of VEGF involves an anti-apoptotic effect that promotes cell survival[21]. In our study, VEGF was dramatically increased by H/R. The VEGF expression in the HPC groups was greater than the H/R group, which revealed that VEGF was involved in anti-apoptosis effect of HPC on MSC. In contrast, we found that CsA had no effect on VEGF expression. Bcl-2 overexpression could enhance VEGF secretion by more than 60% under hypoxic conditions[22].

By contrast, although CsA could reduce MSC apoptosis, stabilize mitochondrial potential, and increase Bcl-2 expression in mitochondria, 0.5 μmol/L CsA did not promote ERK and Akt phosphorylation. This may be the difference in the protective effects between HPC and CsA. HPC is likely to manipulate multiple stress-responsive signaling pathways that contribute to the regulation of MSC apoptosis.

The application of brief, transient periods of non-lethal hypoxia before a subsequent lethal episode of hypoxia markedly delays the development of programmed cell death. The time of hypoxia preconditioning is a critical factor. Prolonged hypoxia failed to exert protective effects and even seemed deleterious in some cells[23]. In our system, hypoxia 10–30 min before lethal insult time-dependently attenuated apoptosis in MSC. Which length of preconditioning time is best in MSC needs further study.

An obvious limitation of the present study was that we used H/R in serum deprivation culture as a simple model to mimic myocardial ischemic and reperfusion, so further *in vivo* studies should be demonstrated.

In summary, the present investigation demonstrates that apoptosis induced by H/R insult was partially attenuated by HPC. Apoptosis suppression by HPC correlated with the prevention of mitochondrial dysfunction and promotion of ERK and Akt phosphorylation and upregulation of Bcl-2 and VEGF expression in H/R-induced apoptosis signaling. The HPC pretreatment prevented the MSC from apoptosis, which could be an effective approach to promote cell transplantation efficiency.

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