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Curcumin pretreatment protects against hypoxia/reoxgenation injury via improvement of mitochondrial function, destabilization of HIF-1α and activation of Epac1-Akt pathway in rat bone marrow mesenchymal stem cells

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ABSTRACT

Bone marrow mesenchymal stem cells (BMSCs) possess promising therapeutic effects and have been considered as a highly desirable agent for tissue injury treatment. However, little survived cells after transplanting due to severe relocated conditions (characterized by prolonged hypoxia and oxidative stress) lead to hampered benefits of BMSCs-based cell therapy. Curcumin, a natural dietary product, has attracted increasing attention owing to its profound pharmacologic properties. Here, we report the protective effects of curcumin pretreatment in BMSCs against hypoxia and reoxygenation (H/R) triggered injury, which mimick ischemia/reperfusion *in vivo*. We found that curcumin pretreatment remarkably inhibited H/R-induced cell viability loss, cell nuclei condensation, LDH leakage, as well as caspase-3 activity increase in BMSCs. Furthermore, curcumin pretreatment prevented H/R-induced mitochondrial dysfunction through expediting adenosine triphosphate production and suppressing reactive oxygen species accumulation and mitochondrial membrane potential decline. In addition, curcumin pretreatment notably induced HIF-1α destabilization, Epac1 and Akt activation, and Erk1/2 and p38 deactivation. However, Epac1 inhibitor ESI-09 obviously restrained the increase of p-Akt induced by curcumin, but not p-Erk1/2 or p-p38, and abrogated the protective effect of curcumin on BMSCs' survival and arrested cell cycle in G0/G1 phase. Taken together, these results demonstrated that curcumin pretreatment conferred BMSCs the ability to survive from H/R injury, which might attribute to its protection on mitochondrial function, destabilization of HIF-1α and activation of Epac1-Akt signaling pathway. Thus, this study provides more pharmacologic aspects of curcumin, and suggests that pre-conditioning of BMSCs with curcumin could serve as an attractive approach for facilitating cell therapy in tissue repair treatment.

1. Introduction

Bone marrow mesenchymal stem cells (BMSCs) have been increasingly indicated as an ideal agent for cell therapy in various diseases such as acute stroke and myocardial infarction [1,2], involving their excellent therapeutic properties such as expandable *ex vivo*, well-characterized differentiation potential, and immunoregulatory effects [3,4]. As the induction of cell therapy is highly dependent on transplanting really enough quantity of cells into the region of interest with maximum preservation of cell viability and function, an important challenge in BMSCs transplantation is the extensive loss of relocated cells in the microenvironment of damaged tissue due to the interplay of ischemia reperfusion, oxidative stress and apoptosis, which largely limits the therapeutic efficacy of BMSCs in tissue repair [5,6].

Hypoxia/reoxygenation (H/R) injury is typically involved in ischemia/reperfusion process, and is closely accompanied with an overload of oxidative stress and mitochondrial dysfunction [7–9]. The reoxygenation that follows hypoxia is suggested to stimulate the bursts of reactive oxygen species (ROS) and the opening of mitonchondrial membrane pore, which not only cause exacerbated oxidative damage central in the pathophysiology of H/R injury but activate signaling paths to induce cell apoptosis as well. Therefore, protection of BMSCs from H/R injury may prove beneficial for cell transplantation.

Hypoxia-inducible factor-1 alpha (HIF-1α) is a key regulator well

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known for governing hypoxia adaption to dictate cell fate in multiple pathologic diseases [10,11]. A significant facet of HIF-1α biology is its activation by, modulation of, hypoxia [12]. Under hypoxia, HIF-1α possesses the ability to promote or resist cell proliferation, which appears to be strongly context-dependent [13,14]; however, its involvement in H/R injury has less been investigated. Exchange protein activated by cAMP-1 (Epac1), also known as a novel sensor of cAMP, is expressed ubiquitously and is essential for numerous critical cellular events such as cell proliferation, migration and adhesion [15]. Epac1 has been implicated in regulating signaling pathways related to cell survival, such as PI3K/Akt activation [16]. Besides, there are growing evidences showing Epac1 involvement in activating Erk1/2 and p38 MAPK cascades in multiple cellular processes [17,18]. However, very little attention has been devoted to Epac1's role in governing above basic signaling inputs to efficiently manage H/R-induced stress.

Emerging evidences have indicated that pharmacologic enhancement of cell bioactivity and function before transplantation could be a valid therapeutic strategy for cell therapy, and would be of great benefit for tissue repair [19,20]. Curcumin, a natural product abundantly in Curcuma longa, is notably implied to act as an anti-apoptotic enhancer or modifier against oxidative insults, which may integrate its anti-oxidative effect and alterations in signal targets [21,22]. Even though many studies have shown the appreciable effects of curcumin on cell protection, little is known regarding the protective effect of curcumin against H/R injury in BMSCs, as well as the underlying intracellular mechanisms. Therefore, in the present study, we aimed to explore the protective effects of curcumin against H/R injury, and the involved roles of mitochondrial function and HIF-1α and Epac1/Akt/MAPK pathways in rat BMSCs.

2. Materials and methods

2.1. Materials

Curcumin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in DMSO to 10 mM as a stock solution. The Epac1 inhibitor ESI-09 was purchased from Selleck Chemicals (Houston, TX, USA) and dissolved in DMSO to make a 10 mM stock solution. The stock solutions were stored at -20℃ and freshly diluted with culture medium to the indicated concentrations before each usage. All the other chemicals and reagents were commercially obtained from Sigma-Aldrich Chemical Company unless otherwise specified.

2.2. Cell culture and hypoxia/reoxygenation (H/R) treatment

Rat bone marrow mesenchymal stem cells (BMSCs) were purchased from Cyagen Biosciences Company (Guangzhou, China) and maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Hyclone, Logan, UT, USA) medium supplemented with 10% fetal bovine serum (FBS; Corning, Manassas, VA, USA) and 100 μg/ml penicillin/streptomycin. The cells were cultured in a humidified atmosphere at 37℃ containing 5% CO_2. For drug treatment, twenty-four hours after cells were cultured in 96-well plates or 6-well plates at a density of 1×10^5/ml in normoxic condition, the culture medium was replaced with fresh complete medium and BMSCs were pretreated with different concentrations of curcumin (1, 5, 10 or 20 μM) for 2 h prior to H/R treatment. For Epac1 blocking experiments, the cells were first incubated with 10 μM ESI-09 for 30 min before curcumin treatment.

To establish the H/R model, cells after different drug treatments were transferred into an incubator chamber (Heal Force, Shanghai, China) filled with 1% O_2, 5% CO_2 and 94% N_2. After hypoxic incubation for the indicated time, BMSCs were moved back to a normoxic incubator (Thermo Scientific, Marietta, Ohio, USA) for reoxygenation and maintained for different time as stated in the following methods. Cells under normoxic condition served as control.

2.3. Cell viability assay

After designated drug treatments, BMSCs in 96-well plates were subsequently exposed to H/R (H/R time: 12/4, 20/4, 36/4 h). Cell viability was determined using a cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Dojindo, Japan). Briefly, each well was added 10 μl of CCK-8 reagent and incubated at 37℃ for 1 h. After shaking for 3 min, the absorbance of each well was recorded on a microplate reader (Tecan, Männedorf, Switzerland) at 450 nm wavelength. Normoxic cells served as control. The viability of BMSCs in each group was presented as percentage of control group. The available H/R time was set according to the results of cell viability.

2.4. Hoechst 33342 staining

BMSCs were cultured on glass coverslips in 12-well plates for 24 h. Following drug treatments, cells were exposed to H/R (20/4 h) and then stained with a Hoechst 33342 staining kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instruction. Briefly, cells were incubated with Hoechst 33342 solution for 10 min at 37℃ and washed twice with PBS. The coverslips were then mounted onto glass slides and observed under the FSX100 Bio Imaging Navigator (Olympus, Center Valley, PA, USA) with 100-fold amplification.

2.5. Lactate dehydrogenase (LDH) release assay

The release of LDH from cultured cells into the supernatants was quantified using a colorimetric assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacture's protocol. Cell supernatants were collected from the cultured cells in 6-well plates after H/R (20/4 h) treatment. LDH activity was measured at 450 nm wavelength on a Tecan microplate reader. The absorbance value of each group was normalized against that of control group.

2.6. Caspase-3 activity assay

Caspase-3 activity was measured using a commercialized caspase-3 activity assay kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instruction. Cell samples were collected from 6-well plates after H/R (20/4 h) treatment. Caspase-3 activity was monitored by the optical density at 405 nm of the released peptide nuclei acid. Protein concentration of the samples was measured by a Bradford protein quantification assay kit (KeyGEN Biotechnology, Nanjing, China). Caspase-3 activity was expressed as the percentage of control group after normalized by protein content.

2.7. Detection of ATP content

Intracellular ATP content was determined using a bioluminescent ATP detection kit (Promega, Madison, WI, USA) following the manufacturer's instruction. Briefly, cells cultured in 96-well plates were subjected to H/R (20/4 h) following drug treatments, and then 100 μl of ATP-releasing reagent was added into each well and the contents were mixed for 2 min on a shaker to induce cell lysis. After incubating in the dark for a further 10 min to stabilize the luminescent signal, the contents were transferred to a black microtiter plate and the bioluminescence was recorded on a Tecan microplate reader based on a luciferin/luciferase assay in the presence of ATP. The bioluminescence intensity of each group was normalized by that of control group and expressed as the relative ATP level.

2.8. Measurement of intracellular ROS production

The production of intracellular ROS was measured with the

molecular probe H_2DCF-DA (D6883, Sigma–Aldrich, St. Louis, MO, USA). Briefly, cells were cultured in black 96-well plates with clear bottom and undertook H/R (20/1 h) treatment. Each well was then loaded with 100 μl of 10 μM H_2DCF-DA in a saline medium containing (in mM): 132.0 NaCl, 4.0 KCl, 1.0 $CaCl_2$, 1.4 $MgCl_2$, 1.2 NaH_2PO_4, 6.0 glucose, 10.0 HEPES (pH 7.4), and incubated in darkness at 37℃ for 30 min. After washing twice with the same saline medium, the DCF fluorescence signal oxidized from H_2DCF-DA was detected using a Tecan fluorescence spectrophotometer at 485 nm (Excitation) and 520 nm (Emission). The fluorescence intensity was expressed as a percentage relative to the control value.

2.9. Mitochondrial membrane potential assay

The change in mitochondrial membrane potential ($\Delta\psi_m$) was assessed using the fluorescent cationic indicator JC-1 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instruction. After H/R (20/4 h) treatment, cells cultured in black 96-well plates with clear bottom or on glass coverslips were treated with JC-1 solution in darkness at 37℃ for 15 min. The plates or coverslips were then washed twice with the incubation buffer. The quantitative intensity of JC-1 fluorescence of cells plated in wells was measured using a Tecan microplate reader (green signal Ex/Em = 480/530 nm, red signal Ex/Em = 530/590 nm). The $\Delta\psi_m$ was expressed as a ratio of the red/green fluorescence intensity normalized to the control fluorescent baseline. The fluorescence micrographs of JC-1 staining of cells grown on coverslips were obtained under an Olympus fluorescence microscope with 100-fold amplification.

2.10. Western blotting

After H/R (20/1 h) treatment, cells in 6-well plates were washed twice with ice-cold PBS and lysed in RIPA buffer (Beyotime) freshly supplemented with 1% (v/v) PMSF and 1% (v/v) protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany) on ice. Cell lysates were separated by centrifugation at 12,000 × *g* for 10 min at 4℃. Protein concentration of the supernatants was determined by a BCA assay kit (Heart Biological Technology Co. Ltd., Xi'an, China). The samples were denatured by mixed with a quarter of 5 × loading buffer and boiled for 10 min. Total protein (20 μg) of each sample was loaded and ran on 10% SDS-PAGE gels, then transferred onto PVDF membrane (Roche, Applied Science, Basel, Switzerland). After blocking with 5% skim milk in T-TBS [0.05% (v/v) Tween-20, 50 mM Tris, 150 mM NaCl, pH 7.5] at room temperature for 1 h, the membranes were incubated with primary antibodies [(Affinity Biosciences, Cincinnati, OH, USA) HIF-1α, AF1009, 1:1000; (Santa Cruz Biotechnology, CA, USA) Epac1, sc-28366, 1:1000; (Cell Signaling Technology, Danvers, MA, USA) p-Akt, #4060, 1:1000; Akt, #4691, 1:1000; p-Erk, #4370, 1:1000; Erk, #4695, 1:1000; p-p38, #4511, 1:1000; p38, #8690, 1:1000; β-actin, #4970, 1:2000; (Huabio, Hangzhou, China) GAPDH, M1211, 1:2000] at 4℃ overnight. On the next day, the membranes were rinsed three times with T-TBS buffer for 10 min each, and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, PA, USA; 1:5000) at room temperature for 1 h. The bands were then developed using ECL western blotting detection reagents (Millipore Corporation, Billerica, MA, USA) and detected by MultiImage Light Cabinet Filter Positions (Alpha Innotech, San Leandro, CA, USA). The intensity of each band was quantified by ImageJ software (NIH, Bethesda, MD, USA).

2.11. Cell cycle assay

Cell cycle progression was examined on a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) using PI/RNase staining buffer (BD Biosciences). In brief, after H/R (20/4 h) treatment, cells in 6-well plates were trypsinized and collected after centrifugation at 1000 rpm for 10 min. The cells were resuspended in cold 75% ethanol and kept at -20℃ for 4 h. After washing twice with PBS to remove the ethanol, the cells were stained with PI/RNase staining buffer at room temperature for 15 min. Flow cytometry analysis was immediately performed using CFlow Plus software (version 1.0; BD Biosciences). Twenty thousand counts were recorded for each sample.

2.12. Statistical analysis

All the values were presented as mean ± SEM and achieved from at least three independent experiments. Student's *t*-test was applied in the comparison between two groups, and one-way ANOVA followed by Turkey's test was used for multiple comparisons between several groups. *P* values less than 0.05 were considered statistically significant. Statistical analysis was done with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. Protective effects of curcumin against H/R-induced injury in BMSCs

In order to find out an optimal H/R treatment duration for BMSCs, we first investigated the effects of selective H/R time episodes (12/4, 20/4, 36/4 h) on cell viability of BMSCs using CCK-8 assay. As shown in Fig. 1A, cell viability was declined with prolonged exposure to hypoxia followed by reoxygenation for 4 h, and remarkably reduced to 82.17% and 73.28% of the control group after 20/4 and 36/4 h H/R treatment, respectively. As exposure to hypoxia for 20 h and reoxygenation for 4 h was the shortest time period when the difference in cell viability compared to control was statistically significant, we decided that hypoxia for 20 h and reoxygenation for indicated time was available for the further study.

Next, we determined the effect of curcumin on BMSCs' viability in this model system. BMSCs were pretreated with different concentrations of curcumin (1, 5, 10, or 20 μM) and then subjected to H/R (20/4 h) injury. Results showed that pretreatment with curcumin (1, 5 and 10 μM) increased cell viability in a concentration-dependent manner, and the protective effect of curcumin pretreatment peaked at the concentration of 10 μM (Fig. 1B); while curcumin at 20 μM caused cell toxicity and was hence not to be used in the following experiments. Our previous study has demonstrated that cell viability of BMSCs was not affected by treatment with curcumin (1–10 μM) alone in normal culture conditions.

Hoechst 33342 staining was performed to evaluate the morphological nuclei changes in BMSCs undergoing H/R. The morphological observations under fluorescent microscopy revealed large, undamaged and diffusely stained cell nuclei in control group, while H/R treatment caused cell nuclei reduction and apoptotic nuclei that were condensed and fragmented. Consistent with cell viability, curcumin pretreatment improved the morphologies of cell nuclei in a concentration-dependent manner, exhibiting more regularly stained cell nuclei of BMSCs (Fig. 1C).

3.2. Curcumin pretreatment reduced LDH release and caspase-3 activity in BMSCs after H/R injury

LDH release from cytoplasm into cell medium was closely correlated with the disruption of plasma membrane during cell injury. We then conducted LDH release assay to explore the effects of curcumin on cellular integrity during H/R by incubating BMSCs with curcumin (1, 5 and 10 μM) before H/R (20/4 h) treatment. BMSCs exposed to H/R exhibited a significant increase in LDH release, whereas LDH leakage from BMSCs pretreated with different concentrations of curcumin decreased in a concentration-dependent manner (Fig. 2A).

Caspase-3 has been known as a key protein in the final process of apoptotic cascade [23]. To find out whether curcumin was able to

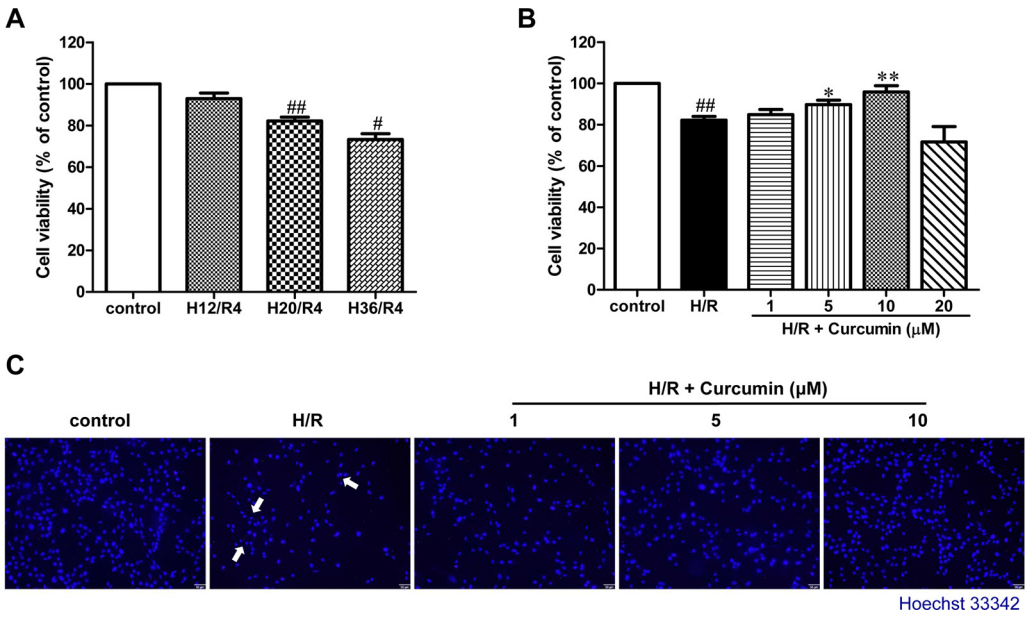

Fig. 1. Protective effects of curcumin pretreatment against H/R-induced cell toxicity in BMSCs. (A) BMSCs were subjected to different durations of hypoxia (12, 20 and 36 h) followed by reoxygenation exposure for 4 h. Cell viability was measured by CCK-8 assay, and hypoxia for 20 h and reoxygenation for 4 h was set as the available time. (B) Cell viability was determined after curcumin pretreatment with H/R (20/4 h) exposure. (C) Morphological nuclei changes of BMSCs after different treatments with Hoechst 33342 staining. White arrows point to condensed cell nuclei. Scale bar = 50 μm. Data were expressed as the relative survival rate of control from three to four independent experiments and as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control; $^{*}P < 0.05$, $^{**}P < 0.01$ versus H/R group.

protect BMSCs from H/R-induced cell apoptosis, we evaluated the caspase-3 activity. Our results showed that the activity of caspase-3 was obviously higher in H/R group than that in control group. In contrast, curcumin pretreatment (5 and 10 μM) could conspicuously decrease caspase-3 activity in BMSCs undergoing H/R injury (Fig. 2B).

3.3. Curcumin pretreatment attenuated mitochondrial dysfunction in BMSCs after H/R injury

To further confirm the protective role of curcumin in our study, we assessed the effects of curcumin on H/R-induced mitochondrial dysfunction by a set of assays. ATP production is an essential indicator for mitochondrial function, as ATP is mainly generated in mitochondria and integrates its role in energy transfer and preservation [24]. The intracellular ATP level was measured based on a luminescence enzymatic reaction. In the present study, ATP level of H/R group was significantly lower than that of control group; however, curcumin pretreatment (10 μM) remarkably reversed the decreased ATP level (Fig. 3A).

Over accumulation of ROS has a pivotal role in disrupting mitochondrial function. A fluorescent assay using the probe H_2DCF-DA was conducted to determine the level of intracellular ROS. We observed that H/R treatment significantly increased the ROS level in BMSCs, whereas pretreatment with 10 μM curcumin prominently reduced the H/R-induced ROS accumulation (Fig. 3B).

We also investigated the effects of curcumin on $\Delta\psi_m$ using JC-1 staining, as decreased $\Delta\psi_m$ causes mitochondrial membrane depolarization and thus initiates an early event of apoptosis cascade [25]. The fluorescent probe JC-1 is potential-dependently accumulated in normal mitochondria with an emission shift from green fluorescences of JC-1 monomers to red fluorescences of JC-1 aggregates [26]. Consequently, the depolarization of $\Delta\psi_m$ was represented by the decreased fluorescence ratio of red to green of JC-1. As shown in Fig. 3C and D, polarized mitochondria in normal BMSCs emitted a red fluorescence after JC-1 staining, while the H/R group exhibited an obvious increase in the amount of green fluorescence and significantly decreased ratio of red/green fluorescence intensity, indicating the dissipation of $\Delta\psi_m$. Conversely, pretreatment with 10 μM curcumin robustly increased red fluorescence and decreased green fluorescence, showing the maintained $\Delta\psi_m$ of BMSCs.

3.4. Effects of curcumin pretreatment on the protein expression of HIF-1α and Epac1, and the activation of Akt, Erk1/2 and p38 in H/R-treated BMSCs

To gain insights into the regulation of involved cell signalings whereby curcumin pretreatment prevented cell toxicity in response to H/R injury, we next explored the effects of curcumin on the specific signaling pathways in BMSCs undergoing H/R. HIF-1α, a major hypoxia stress-response transcription factor, is also reported to be regulated by ROS [14]. Immunoblotting results showed that hypoxia for 20 h and reoxygenation for 1 h significantly elevated HIF-1α protein expression in BMSCs, while curcumin pretreatment (10 μM) led to a significant drop in HIF-1α protein level (Fig. 4A). We also determined whether Epac1-dependent signal transduction pathways were involved in the protective effects of curcumin, as Epac1 and its downstream effectors

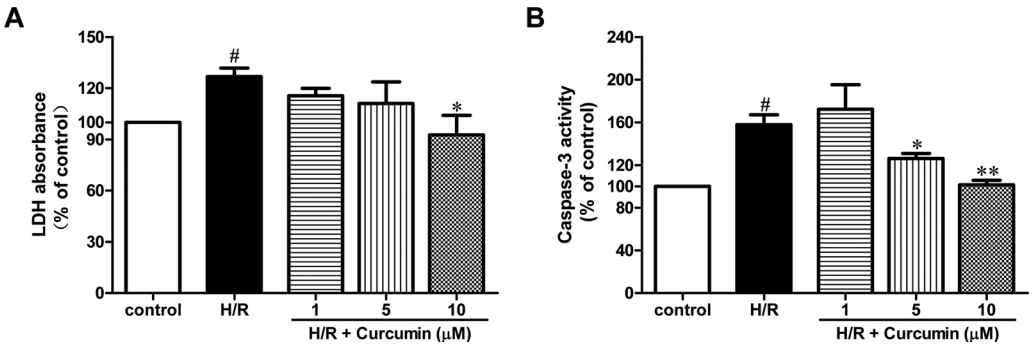

Fig. 2. Curcumin pretreatment reduced LDH leakage and caspase-3 activity in H/R-treated BMSCs. (A) The release of LDH from cytosol into culture medium was determined by a colorimetric assay ($n = 4$). (B) Caspase-3 activity in BMSCs ($n = 3$). Data, normalized by control group, were shown as mean ± SEM. $^{\#}P < 0.05$ versus control; $^{*}P < 0.05$, $^{**}P < 0.01$ versus H/R group.

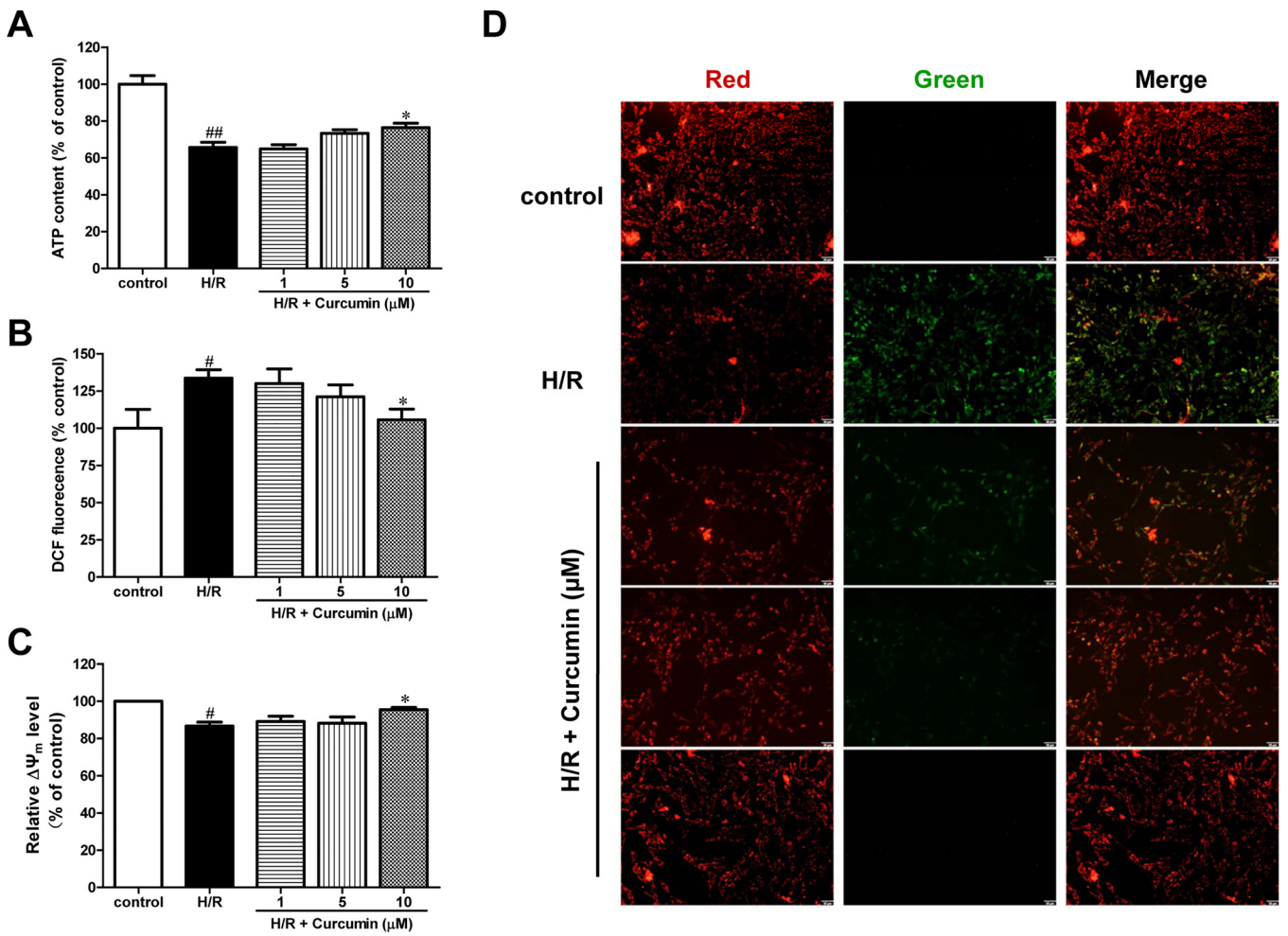

Fig. 3. Curcumin pretreatment ameliorated H/R-induced mitochondrial dysfunction in BMSCs. (A) ATP contents were measured by a luminescence enzymatic assay kit ($n = 4$). (B) ROS accumulation was assessed using the molecular probe H_2DCF-DA ($n = 5$). (C) Quantitative analysis of mitochondrial membrane potential with JC-1 staining ($n = 4$). (D) Representative morphological images of JC-1 staining. Scale bar = 50 μm. Data were presented as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control; $^{*}P < 0.05$ versus H/R group.

have been demonstrated to play an important role in cell protection [16]. As shown in Fig. 4B-D, H/R treatment remarkably reduced Epac1 protein expression, which was in parallel with reduced phosphorylation of Akt and enhanced phosphorylation of Erk1/2 and p38. In contrast with H/R-treated BMSCs, pretreatment with 10 μM curcumin robustly elevated Epac1 protein level and promoted Akt phosphorylation, as well as suppressed Erk1/2 and p38 phosphorylation.

3.5. Effects of Epac1 inhibition on the activation of Akt, Erk1/2 and p38 induced by curcumin in BMSCs after H/R injury

ESI-09, a specific inhibitor of Epac1, was employed in the study to further verify if Epac1 inhibition would affect the phosphorylation of Akt, Erk1/2 and p38 induced by curcumin. It was found that ESI-09 radically abrogated curcumin-induced increase in phosphorlated Akt (p-Akt), but p-Erk1/2 or p-p38 level was not markedly influenced in BMSCs after H/R injury (Fig. 5A-C). These results suggested that activation of PI3K/Akt by curcumin pretreatment in H/R-treated BMSCs was, at least partially, ascribed to the up-regulation of Epac1 signaling.

3.6. Blockade of Epac1 abrogated the protective effects of curcumin in BMSCs during H/R injury

To further demonstrate the involvement of Epac1 in curcumin-induced protection against H/R injury in BMSCs, cell viability and cell cycle assay were performed with the use of ESI-09. Strikingly, results showed that ESI-09 led to significant decrease of cell viability in curcumin-pretreated BMSCs during H/R injury (Fig. 6A). In parallel with cell viability, cell cycle analysis also provided evidence that blockade of Epac1 before curcumin pretreatment arrested more cells in G0/G1 phase and decreased S-phase fraction of BMSCs, without affecting G2/M phase, when compared with curcumin preteatment group in the process of H/R (Fig. 6B). Above results collectively confirmed the participation of Epac1 in curcumin-induced BMSCs survival against H/R injury.

4. Discussion

In recent two decades, BMSCs transplantation therapy has been expected to bring considerable benefits to a wide range of tissue regeneration and repair in both animal and clinical studies [27,28]. However, it is worth to be noted that the reported therapeutic effects are rather restricted, at least in part, due to the limited cells survived after relocating to damaged tissue sites. Under the harsh microenvironment of ischemic tissue featured by hypoxia and free radical damage, BMSCs mostly fail to exert their compensatory functions [5,6]. As an efficient modality to enhance the biological activity of implanted cells under pathologic conditions, preconditioning cells with drugs has been shown to potentiate the survival ability of incorporated BMSCs in hostile microenvironments [19]. Curcumin, also known as an

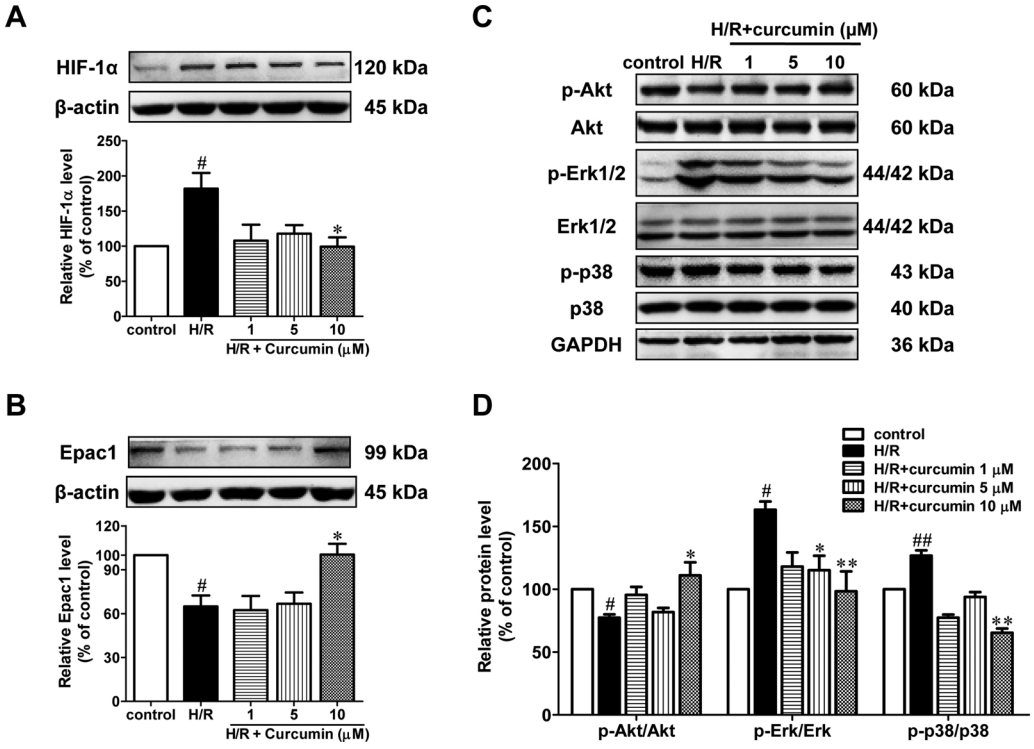

Fig. 4. The protein expression of HIF-1α, Epac1, as well as Akt, Erk1/2, p38 and their corresponding phosphorylated forms following H/R treatment in curcumin-pretreated BMSCs. (A–B) Immunoblotting analysis on the protein expression of HIF-1α (A) and Epac1 (B) in curcumin-pretreated BMSCs followed by H/R (20/1 h) exposure, with β-actin as loading control. (C–D) Immunoblots (C) and bar graphs (D) showing the protein level changes of p-Akt and Akt, p-Erk1/2 and Erk1/2, as well as p-p38 and p38, with GAPDH as loading control. Each data point was normalized against its corresponding loading control or total protein level, and was expressed in percentage of control as mean ± SEM (n = 3–4). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control; $^{*}P < 0.05$, $^{**}P < 0.01$ versus H/R group.

antioxidant, provides widely anti-oxidative and anti-apoptotic functions in various cells and tissues [22]. However, there is currently unknown about the functional role of curcumin preconditioning in BMSCs during H/R injury mimicking the ischemic/reperfusion event *in vivo*, let alone the underlying mechanisms. In this study, curcumin preconditioning was for the first time shown to confer BMSCs resistance to survive from H/R injury via enhancement of mitochondrial function, destabilization of HIF-1α and activation of Epac1-Akt signaling pathway.

We first imposed different sequential hypoxia and reoxygenation episodes to BMSCs on the basis of several referential literatures, and an optimal H/R treatment duration was selected according to cell viability results (Fig. 1A). The concentration and pretreatment duration of curcumin were also cautiously selected based on our previously published studies and pilot experiments in the current study. Intriguingly, we found that pretreatment of BMSCs with curcumin prevented H/R-induced cell viability reduction, and also improved nuclei morphology in a concentration-dependent manner (Fig.1B and C). Notably, the protective effects of curcumin reached the peak at the concentration of 10 μM in our experimental situation.

LDH leakage from cytosol into culture medium is an important index for the extent of cell membrane integrity rupture [29]. Caspase-3 exerts crucial roles in initiating cell apoptosis in diverse cell injury processes [23]. BMSCs subjected to H/R showed significantly increased LDH release and a remarkably higher level of caspase-3 activity. Strikingly, pretreatment of BMSCs with curcumin mitigated LDH

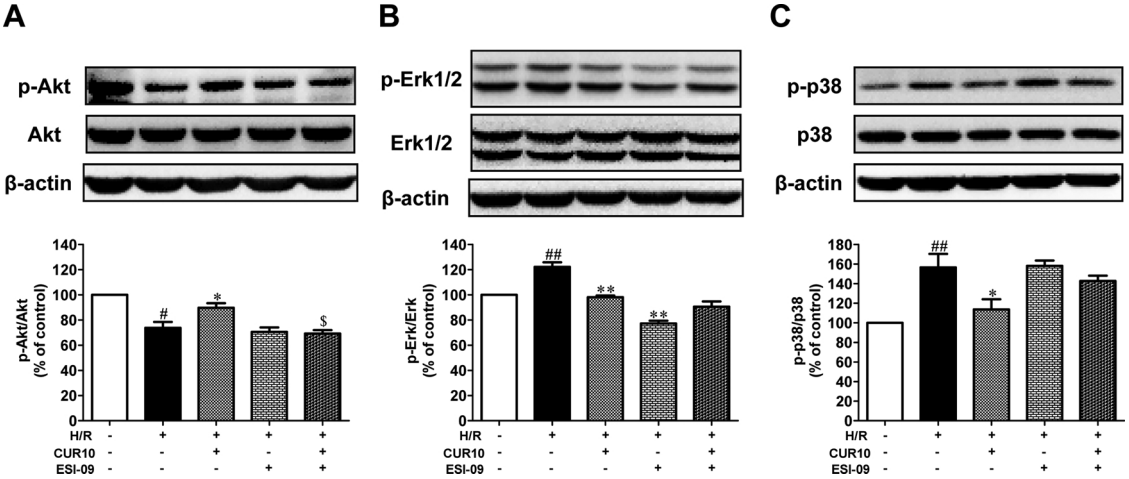

Fig. 5. Inhibition of Epac1 abrogated the up-regulated phosphorylation of Akt in curcumin-pretreated BMSCs during H/R injury. BMSCs were pretreated with 10 μM ESI-09 for 30 min followed by 2 h of 10 μM curcumin (CUR10) treatment, and then exposed to H/R (20/1 h) injury. (A–C) Immunoblotting analysis on the protein level changes of p-Akt and Akt (A), p-Erk1/2 and Erk1/2 (B), as well as p-p38 and p38 (C) in BMSCs following the indicated treatments. Bar graphs on the down column showing the differences after each data point of the phosphorylated form was normalized against its corresponding total protein level with the value in control was adjusted to 100%. β-actin served as loading control. Error bars represented mean ± SEM (n = 3–4). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control; $^{*}P < 0.05$, $^{**}P < 0.01$ versus H/R group; $^{\$}P < 0.05$ versus curcumin + H/R treatment group.

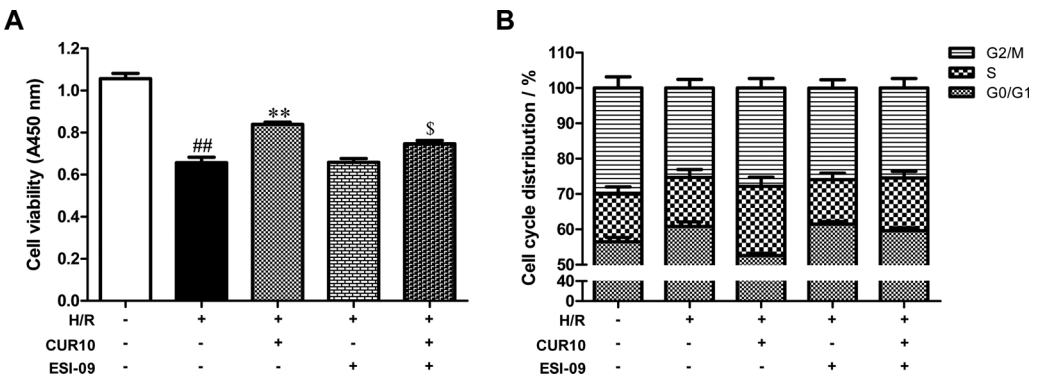

Fig. 6. Epac1 blockade reversed the protective effects of curcumin pretreatment on cell viability and cell cycle progression in H/R-treated BMSCs. (A) Cell viability was measured by CCK-8 assay. (B) Cell cycle distribution was assessed by flow cytometry using PI/RAase staining. Data were presented as mean ± SEM (n = 3–6). $^{\#\#}P < 0.01$ versus control; $^{**}P < 0.01$ versus H/R group; $^{\$}P < 0.05$ versus curcumin + H/R treatment group.

release and reduced caspase-3 activity (Fig. 2A and B). These results provide favorable aspects for curcumin pretreatment in protecting BMSCs from H/R-induced cell injury and elicit further evidence supporting the anti-apoptotic role of curcumin.

Mitochondrial dysfunction plays an essential role in cell apoptosis during H/R injury [30]. ROS, which are mainly produced during normal mitochondria oxygen metabolism and function as essential signaling molecules, have been found to be largely responsible for mitochondrial dysfunction [7,8]. Under H/R, over-produced ROS could cause direct disruption of mitochondrial membrane permeability. $\Delta\psi_m$ is a crucial marker for mitochondrial function, and its steady state is highly dependent on mitochondrial membrane integrity. Excessive ROS instigate the opening of mitochondrial permeability transition pore and thus lead to a decline in $\Delta\psi_m$ [31]. Moreover, mitochondrion is a key sub-organelle closely related to cell energy production. The destructed transmembrane electrochemical gradient eventually reduced ATP generation [32]. In line with these notions, H/R exposure caused obviously diminished ATP generation in BMSCs, and this was largely related to a sustained high level of intracellular ROS and the dissipation of $\Delta\psi_m$ (Fig. 3A-C). More importantly, our results showed that curcumin pretreatment enhanced ATP level, which was accompanied by less ROS formation and attenuated $\Delta\psi_m$ decline. The involvement of mitochondrial protection appears to be an important approach by which curcumin conferred beneficial effects on BMSCs against H/R injury.

HIF-1α is a master regulator for cell responses to low oxygen conditions in many disease settings, and is normally sequestered in the cytoplasm through ubiquitination-dependent degradation under normoxic conditions [10,11]. Toward hypoxia activation, HIF-1α is retained in the cytoplasm and translocates to the nucleus to activate the expression of hypoxia-inducible genes, but the long sustained up-regulation of HIF-1α has been recently reported to turn on the process of cell death. Typically, HIF-1α-induced apoptosis is most common under quite low oxygen concentrations or prolonged hypoxia conditions [33,34]. Likewise, in our study, BMSCs were exposed to a severe and long period of hypoxia (at 1% O_2 for 20 h). This condition led to pro-apoptotic HIF-1α accumulation and apoptosis stimulation, whereas curcumin pretreatment abolished HIF-1α induction by H/R and rescued cell damage (Fig. 4A). Notably, curcumin pretreatment did not affect HIF-1α mRNA level in BMSCs in our study (data not shown), suggesting that curcumin regulates the stability of HIF-1α. Our data are in agreement with previous studies supporting the notion that HIF-1α's stabilization or its delayed degradation under severe and prolonged H/R condition plays an important role in triggering cell death.

Epac has been identified as a new target of cAMP in signal transduction and exists in two isoforms, Epac1 and Epac2. Epac1 is ubiquitously expressed, while Epac2 is predominantly distributed in brain, kidney and pancreas [15]. Increasing evidences indicate that Epac1 integrates its role into intracellular signaling networks and regulates a wide variety of biologic responses and functions such as cell proliferation. Most biologic effects of Epac1 require its exchange activity towards the Ras family members, thereby inducing activation of a number of diverse molecular mediators, including PI3K/Akt and certain members of MAPK cascades [16]. Our study revealed that H/R treatment significantly reduced the protein expression of Epac1 in BMSCs, while this protein level was prominently enhanced in curcumin-pretreated BMSCs after H/R injury (Fig. 4B), suggesting the probable involvement of Epac1 signaling route in supporting the protective effects of curcumin in H/R-treated BMSCs. There are growing awarenesses of the pivotal influences of PI3K/Akt and Erk1/2 and p38 MAPK in dictating whether cells will ultimately defeat or succumb to H/R damage [35,36]. In this study, we have identified that Akt was rapidly deactivated, but Erk1/2 and p38 were robustly activated in BMSCs after H/R treatment. Conversely, curcumin pretreatment strikingly enhanced p-Akt level and restrained the phosphorylation of Erk1/2 and p38 (Fig. 4C and D). Epac1 regulating the phosphoryalation of Akt and Erk1/2 and p38 is extensively involved in many eukaryote behaviors, like proliferation and migration, but if it plays further roles in these signal molecules' activity in curcumin-pretreated BMSCs undergoing H/R has been less known. Thus, based on above findings, we were eager to reveal if Akt, Erk1/2 and/or p38 signals are probably involved in Epac1 signaling transduction pathway in our study. Results showed that putative inhibition of Epac1 by ESI-09 completely abolished the increase of p-Akt level, but p-Erk1/2 or p-p38 level was not markedly affected (Fig. 5A-C), suggesting that Epac1 account, at least to some extent, for the activation of Akt in curcumin-pretreated BMSCs under H/R. Also, Epac1 blockade by ESI-09 resulted in significantly retarded cell survival, and arrested much more cells in G0/G1 phase (Fig. 6A and B). These findings collectively suggest that Epac1-Akt signaling activation might be a critical mechanism for curcumin-induced enhancement of cell survival in H/R-treated BMSCs. Given that ESI-09 has been revealed to compete for the same binding domain on Epac1 protein with cAMP [37], it is possible that ESI-09 manages to displace cAMP from Epac1 protein and leads to an transient increase of endogenous cAMP level which then, in turn, activates other yet to be identified regulatory partners, such as PKA, CREB, which may mainly govern the phosphorylation of Erk1/2 and p38. Nonetheless, further investigations are warranted to delineate additional phosphorylation events and the signaling inputs from downstream molecules that are involved in mediating protective effects of curcumin in BMSCs.

In conclusion, our study has demonstrated that curcumin pretreatment-induced improvement of mitochondrial function, destabilization of HIF-1α and activation of Epac1-Akt signaling produce synergistically protective effects on BMSCs' survival under H/R condition. These results suggest that curcumin pretreatment could be a promising approach to potentiate the survival of transplanted BMSCs for tissue repair.

Conflict of interest

The authors declare no conflict of interests with this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.11.005.

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