Hypoxia Induces Autophagy of Bone Marrow-Derived Mesenchymal Stem Cells via Activation of ERK1/2

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Abstract

Background: Bone marrow-derived mesenchymal stem cells (bmMSCs) are the most promising seed cells for cell transplant therapy. Hypoxia is a known stimulus of autophagy. Recent studies showed that hypoxia promotes autophagy of human placental chorionic plate-derived mesenchymal stem cells (CP-MSCs). However, whether hypoxia affects autophagy of bmMSCs has not been examined. The goal of this study was to investigate the effect of hypoxia on autophagy of mouse bmMSCs and the underlying mechanisms. Methods: BmMSCs from mouse bone marrow were randomly divided into three groups: control (C), hypoxia (H) and hypoxia + reoxygenation (H+R) groups. Subsequent autophagic signals were analyzed by immunostaining and Western blot assays. Results: The expression of autophagic signals LC-3, Atg5 and Beclin-1, as well as the conversion of LC3B-I to LC3B-II in bmMSCs were significantly increased in H group as compared with control (p<0.05). These autophagic signals were also higher in H+R group than in H group (p<0.05). Notably, application of ERK1/2 inhibitor U0126 (5μM) significantly repressed hypoxia-induced expression of LC-3 and Atg5, as well as conversion of LC3B-I to LC3B-II (p<0.05). Conclusion: Hypoxia can induce autophagy of bmMSCs, which depends on activation of ERK1/2 pathway.

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Introduction

Bone marrow-derived mesenchymal stem cells (bmMSCs) are multipotent adult stem cells capable of differentiating into diverse cell types, including osteocytes, adipocytes, endothelial cells, cardiomyocytes and neurons when exposed to the appropriate conditions [1]. Currently, bmMSCs have been the most promising candidates for tissue engineering and regenerative medicine. Clinical and experimental studies have shown that bmMSCs have the potential to treat a wide range of diseases, such as neurodegeneration, stroke and heart infarction [2-4].

Autophagy is a cell homeostatic process that involves degradation and recycling of unnecessary and dysfunctional cellular components through the actions of lysosomes [5]. It is important for balancing sources of energy in the development process and can be induced in many physiological and pathological states [6]. Autophagy protects against genesis of many diseases through selectively eliminating the misfolded and aggregated proteins, as well as the damaged cellular organelles such as mitochondria, endoplasmic reticulum and peroxisomes [6]. The balance between autophagy and apoptosis determines cell survival [5]. It has been reported that well-controlled hypoxia (0.1-3% oxygen) causes cell autophagy and survival, however, severe hypoxia or anoxia (<0.1% oxygen) often leads to cell apoptosis [7].

Previous studies have demonstrated that hypoxia induces autophagy in diverse cell types such as endothelial cells, cardiomyocytes, fibroblasts and tumor cells [5, 8-10]. These studies have indicated that hypoxia-inducible factor-1 α (HIF-α) is the main mediator for hypoxia-induced cellular autophagy [9,10]. Extracellular signal-regulated kinase 1/2 (ERK1/2) are the upstream signals of HIF-α. Their activation participates in regulating the activity of HIF-α [11]. Previous studies have indicated that hypoxia can activate ERK1/2 in many cell lineages [12].

A recent study showed that hypoxia promotes autophagy of human placental chorionic plate-derived mesenchymal stem cells [13], but the underlying mechanisms are unclear. Meanwhile, the effect of hypoxia on autophagy of bmMSCs is still not elucidated. In this study, we investigated the effect of hypoxia on autophagy of bmMSCs and its relations to activation of ERK1/2.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from GIBCO/Life Sciences (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Bioengineering Material Co., Ltd. (Hangzhou, Zhejiang, China). PE-conjugated rabbit anti-mouse CD44, FITC-conjugated rabbit anti-mouse CD90, rabbit anti-mouse LC3B-I/II, rabbit anti-mouse Atg5, rabbit anti-mouse Beclin-1, rabbit anti-mouse phospho-ERK1/2, rabbit anti-mouse ERK1/2 and goat anti-mouse β-actin primary antibodies, as well as the secondary antibodies were purchased from Abcam (Cambridge, MA, USA). ProlongH Gold antifade reagent with DAPI was obtained from Invitrogen/life technologies (Carlsbad, CA, USA). U0126 was purchased from Sigma-Aldrich (StLouis, MO, USA).

Isolation and culture of bmMSCs

BmMSCs were isolated and cultured as recently published protocols [1]. The cells were cultured in DMEM supplemented with 15% FBS, 2mM L-glutamine, 100U/mL penicillin and 100g/mL streptomycin in a CO₂ incubator. The third passage bmMSCs were used in the following experiments. The use of animals and study protocols were approved by the Ethics Committee of Xinxiang Medical University.

Experiment design and hypoxia treatments

BmMSCs were randomly divided into three groups: control (C), hypoxia (H) and hypoxia + reoxygenation (H+R) groups. They were plated in 6-well or 24-well plates with/without round coverslips.
When the cells in H and H+R groups reached 70% confluence, they were put in a hypoxia chamber. The chamber was tightly closed and imported with 95% N₂ and 5% CO₂ thrice to replace the air, and then put a 37°C incubator for 6 hours. The cells in H group were directly used to do immunofluorescence staining or extract proteins after exposure to hypoxia; the cells in H+R group were cultured in a CO₂ incubator for additional 1 hour for reoxygenation following hypoxia; the cells in control group were cultured in a CO₂ incubator at all times.

**Immunostaining**

BmMSCs grown on 10 mm round coverslips were fixed with 4% buffered paraformaldehyde for 15 min, and subsequently treated with 0.1% Triton X-100 in PBS for 10 min at room temperature. And then, the cells were blocked with 1% BSA/10% goat serum in PBS, and incubated with PE-conjugated CD44 antibody (1:200) at 4°C for 1 hour. After washing thrice with PBS, the cells were incubated with FITC-conjugated CD90 antibody (1:200) at 4°C for additional 1 hour. After washing with PBS and water, the coverslips were mounted on slides using ProlongH Gold antifade reagent with DAPI and imaged with a fluorescence microscope.

For analysis of LC-3 and Atg5 expression, cells were firstly treated with hypoxia and reoxygenation, and then fixed and blocked as above-mentioned protocols. The cells were incubated with rabbit anti-mouse LC-3 antibody/rabbit anti-mouse Atg5 antibody, and subsequently incubated with duck anti-rabbit TR-conjugated secondary antibody/duck anti-rabbit FITC-conjugated secondary antibody.

**Western blot**

Proteins were extracted from bmMSCs and separated by 12% SDS-PAGE. After electrophoresis, proteins were transferred to the PVDF membranes. The membranes were blocked with 5% BSA in TBS-T, and then incubated with LC3B-I/II, Agt5, Beclin-1, phospho-ERK1/2, ERK1/2 and β-actin primary antibodies at 4°C overnight. And then, the blots were incubated with HRP-conjugated second antibodies for 1 hour at room temperature. The immunoreactive bands were visualized with enhanced chemiluminescence.

**Statistical analysis**

Statistical analysis was performed with SPSS16.0 software. Data were presented as the mean ± standard deviation (SD). Univariate comparisons of means were evaluated using the Student \( t \) tests and/or one-way ANOVA with Tukey’s post-hoc adjustment for multiple comparisons when appropriate. \( P<0.05 \) was considered statistically significant difference.

**Results**

**Identification of bmMSCs**

As shown in Fig. 1, bmMSCs displayed long-spindle and typical fibroblast-like shapes with positively expressing MSC specific surface markers CD44 and C90.

**Hypoxia increases LC-3 expression in bmMSCs**

Microtubule-associated protein 1 light chain-3 (LC-3) is an important component of autophagosomal membrane, and its expression levels are positively correlated with cellular autophagic activity [14]. In this study, LC-3 expression was analyzed by immunofluorescence staining and Western blot. As shown in Fig. 2A and B, immunofluorescence data showed that hypoxia significantly increased LC-3 expression in bmMSCs (p<0.05, vs. control); reoxygenation following hypoxia (H+R) could further enhance LC-3 expression (p<0.05, vs. hypoxia). Western blot data showed a marked increase of LC3B-II/LC3B-I (conversion of LC3B-I to LC3B-II) in H group as compared with control group (p<0.05). Furthermore, LC3B-II/LC3B-I was also markedly higher in H+R group than in H group (p<0.05). The conversion of LC3B-I to LC3B-II is a distinctive indicator of autophagosome formation [15].

**Hypoxia increases Atg5 and Beclin-1 expression in bmMSCs**

Cellular autophagic responses depend on autophagy protein 5 (Atg5) [15]. As shown in Fig. 3A and B, immunofluorescence showed that Atg5 expression was markedly increased
in hypoxia (H) group as compared with control (p<0.05); and it was further enhanced by reoxygenation (vs. H group, p<0.05). These data were confirmed by Western blot assay (Fig. 3C).

Beclin-1 is required for Atg5-mediated autophagy [16]. Western blot assay showed that the change pattern of Beclin-1 was same as LC-3 and Atg5 (Fig. 4A).

Fig. 1. Immunofluorescence shows that bmMSCs are positive for MSC specific surface markers CD44 (red fluorescence) and C90 (green fluorescence).

Fig. 2. LC-3 expression in bmMSCs after exposure to hypoxia and reoxygenation. A. Immunostaining shows LC-3 expression in bmMSCs. B. Quantification of fluorescence density of LC-3. C. Western blot assay shows LC3B-I and LC3B-II expression and conversion of LC3B-I to LC3B-II in bmMSCs. Bar graphs represent mean ± SD (n = 5 per group). *P<0.05 vs. control (C) group; #P<0.05 vs. hypoxia (H) group.
Hypoxia increases phospho-ERK1/2 expression

Hypoxia is a strong inducer for activation of ERK1/2, and the activated ERK1/2 participate in regulating hypoxia-induced cellular autophagy [17, 18]. In this study, we found that phospho-ERK1/2 were markedly upregulated in hypoxia-treated bmMSCs (p<0.05, vs.
control), which showed that ERK1/2 were activated in bmMSCs after exposure to hypoxia (Fig. 4B). The expression of phospho-ERK1/2 was further increased by reoxygenation (in H+R group) (p<0.05, vs. H group; Fig. 4B). Of note, hypoxia and reoxygenation did not affect the total ERK1/2 expression (Fig. 4B).

**ERK1/2 inhibitor U0126 inhibits hypoxia-induced autophagy**

To further delineate the role of ERK1/2 in hypoxia-induced bmMSC autophagy, we applied ERK1/2 inhibitor U0126 (5μM) as the cells were exposed to hypoxia plus reoxygenation (H+R). Immunofluorescence data showed that U0126 significantly inhibited expression of LC-3 and Atg5 (Fig. 5 A-D; p<0.05, vs. H+R). Western blot data also showed that U0126 inhibited hypoxia-induced conversion of LC3B-I to LC3B-II (increase of LC3B-II/LC3B-I) and Atg5 expression (Fig. 5E-F; p<0.05, vs. H+R).

**Discussion**

In this study, we for the first time show that hypoxia promotes autophagy of bone marrow-derived mesenchymal stem cells (bmMSCs), which is involved in activation of ERK1/2. Application of ERK1/2 inhibitor U0126 significantly inhibits hypoxia-induced autophagy in bmMSCs.

BmMSCs have been widely used in tissue regenerative medicine. The survival of bmMSCs in the receipts after transplantation is the most important factor determining the efficiency of transplant therapy. Autophagy contributes to cell survival [19]. In this study, we found that hypoxia stimulated autophagic responses in bmMSCs. The expression of autophagic markers LC-3, Atg5 and Beclin-1, as well as the conversion of LC3B-I to LC3B-II were markedly increased in bmMSCs after exposure to hypoxia for 6 hours. Reoxygenation following hypoxia could further enhance expression of autophagic signals and conversion of LC3B-I to LC3B-II.
LC3 is a typical marker of autophagy which participates in the formation of autophagosomes [14]. The activity of cellular autophagy can be indicated by LC3 expression and location. During the formation process of autophagosome, LC3B-I incorporates into autophagosomal membrane, and converts to LC3B-II [20]. Thus, the conversion of LC3B-I to LC3B-II also reflects the activity of cellular autophagy. Autophagy protein 5 (Atg5) is necessary for autophagy due to its role in autophagosome elongation. Atg5 can be activated by Agt7, and then forms a complex with Agt12. The Agt5-Agt12 complex modulates the conversion of LC3B-I to LC3B-II [21, 22]. Beclin-1 is another key regulator of cellular autophagy. Atg5-mediated autophagy depends on activation of Beclin-1 [16].

ERK1/2 are a couple of protein kinases that are widely expressed in mammalian cells, and participate in regulating a series of cellular functions such as cell proliferation, differentiation, migration and apoptosis [23]. Diverse stimuli, including mitogens, cytokines, infections and growth factors, can activate ERK/2. Previous studies demonstrated that hypoxia activates ERK1/2 in many cell types [12]. The activation of ERK1/2 has been known to stimulate autophagic responses in tumor cells and other cell lineages [24]. In the present study, we found that hypoxia also caused an activation of ERK1/2 (increase of phospho-ERK1/2 expression) in bmMSCs. Reoxygenation following hypoxia could further increase expression of phospho-ERK1/2. More importantly, application of ERK1/2 inhibitor U0126 significantly repressed hypoxia-induced autophagic responses in bmMSCs. These data suggested that hypoxia/reoxygenation-mediated autophagy in bmMSCs is at least partially dependent on activation of ERK1/2.

In summary, the present study demonstrates that hypoxia alone and combination of hypoxia and reoxygenation both promote autophagic responses in bmMSCS, which depends on activation of ERK1/2.

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Disclosure Statement

None.

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