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Effect of Sustained Hypoxia on Autophagy of Genioglossus Muscle-Derived Stem Cells

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Background: Previous studies have demonstrated that sustained hypoxia in people with obstructive sleep apnea (OSA) impairs upper airway muscle activity, but the underlying mechanism remains poorly understood. As autophagy acts as an important regulator under hypoxia stress, we performed an *in vitro* investigation of the effects of sustained hypoxia on autophagy of genioglossus muscle-derived stem cells (GG MDSC), an important component of the upper airway muscle.

Material/Methods: Genioglossus MDSCs were obtained from Sprague-Dawley (SD) rats and identified by using immunofluorescence staining for CD34, Sca-1, and desmin. GG MDSCs were incubated under normoxic or sustained hypoxic conditions for different periods of time. Western blotting was used to detect LC3 and Beclin 1, which are 2 important proteins in autophagy flux, and autophagolysosomes accumulation was observed by transmission electron microscopy (TEM). The mRNA and protein levels of HIF-1α and BNIP3 were evaluated by RT-PCR and Western blot analysis, respectively.

Results: Our study shows that sustained hypoxia promotes the expression of LC3BII and Beclin 1 in GG MDSCs in a time-dependent manner. TEM showed an increased number of autophagolysosomes in GG MDSCs under sustained hypoxia for 12 and 24 h. In addition, hypoxia activated the HIF-1α/BNIP3 signal pathway both at protein levels (shown by Western blot) and at mRNA levels (shown by RT-PCR).

Conclusions: Our study shows that sustained hypoxia promotes autophagy in GG MDSCs, and the HIF-1α/BNIP3 signal pathway was involved in this process.

MeSH Keywords: Autophagy • Cell Hypoxia • Hypoxia-Inducible Factor 1, alpha Subunit • Sleep Apnea, Obstructive

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Background

It has been well established that dysfunction of upper airway dilator muscles is closely involved in the pathophysiology of obstructive sleep apnea (OSA) [1], and among them, genioglossus (GG) is the most important pharyngeal dilator muscle in the maintenance of upper airway patency [2]. Previous studies have shown that sustained hypoxia in people with OSA impairs upper airway muscle activity [3]. Depression of GG activity is associated with hypoxic ventilatory depression (HVD), which arises in the late phase of the biphasic ventilatory response during sustained hypoxia [4].

Muscle-derived stem cells (MDSCs) are earlier progenitors than satellite cells and other myogenic cells found within skeletal muscle, and they possess a higher regeneration capacity and a broader range of multilineage capabilities [5]. The literature on GG MDSCs is scant, and how hypoxia influences GG MDSCs and the mechanism involved are unclear.

Autophagy, meaning "self-eating", is a lysosomal degradative process of the misfolded or unnecessary proteins and damaged organelles [6]. Basal autophagy serves as a cellular housekeeper and is essential for the homeostasis of cells. Autophagy is activated when cells experience stress such as nutrient deprivation, hypoxia, DNA damage, or intracellular pathogens [7]. It has been well established that autophagy acts as an important regulator during hypoxia stress [8]. Hypoxia-induced autophagy has been considered as a cell survival and cytoprotective process [9]. As GG activity declined when exposed to sustained hypoxia [4], we explored the role of autophagy in sustained hypoxia-induced GG damage.

In this study, we investigated the effect of sustained hypoxia on autophagy of GG MDSCs. We also explored the molecular regulatory mechanisms of sustained hypoxia-induced autophagy of GG MDSCs.

Material and Methods

Animals

Sprague-Dawley (SD) rats were provided by the Laboratory Animal Center of the Medical College of Shandong University. All procedures in this study were approved by the Animal Care Committee of Shandong University (Ji'nan, China).

Cell culture of genioglossus muscle-derived stem cells (MDSCs)

Animals were euthanized under sterile conditions. Muscle tissues were dissected, minced, and separated from connective tissue, rinsed with Hank's solution, and transferred to centrifuge tubes. Successive enzymatic digestion was performed with 0.05% collagenase type II for 40 min and 0.25% trypsin-EDTA for 30 min at 37°C with continuous slow shaking and was stopped with 20% fetal bovine serum. After 75-μm sieve filtration, the cells were collected, centrifuged at 1000 rpm for 1 min, resuspended in DMEM-F12 with 20% fetal bovine serum, and plated in a culture flask with proliferation medium (DMEM-F12, 20% fetal bovine serum, and 1% penicillin-streptomycin). After 2 h, nonadherent cells were transferred to a new flask. After 24 h, and every 24 h for the next 5 days, the same procedure of transferring nonadherent cells was repeated. Finally, the last isolated cells isolated were accepted MDSCs. At 80% confluence, the cells were dissociated with trypsin/EDTA, replated at a cell density of $1.0–2.5\times10^3$ cells/cm^2 and cultured for 3–4 weeks before performing the cell treatments described below. Normoxic genioglossus MDSCs were cultured in a normal incubator (21% O_2, 5% CO_2; 74% N_2). Hypoxic genioglossus MDSCs were cultured in a hypoxia chamber with an atmosphere of 1% O_2, 5% CO_2, and 94% N_2.

Immunofluorescence staining

Cells were seeded at 10 000 cells/well in 12-well cell culture plates, and incubated for 24 h under normoxic or hypoxic conditions. Cells were washed 3 times with PBS for 5 min each time. Then, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.2% Triton X-100 for 10 min. Consequently, cells were washed with PBS and blocked with 2.5% BSA for 60 min. The primary antibodies were diluted with PBS containing 3% BSA as follows: rabbit anti-CD34 (1: 100; Abcam, USA), rabbit anti-Sca-1 (1: 200; Eterlife, UK), rabbit anti-desmin (1: 50; Abcam, USA), and rabbit anti-CD45 (1: 100; Abcam, USA). The nuclei were counterstained with DAPI (Beijing ComWin Biotech Co., LTD., China) for 10 min. The coverglass was observed and photographed to show representative cells using a fluorescent microscope (TE2000; Nikon Corporation, Tokyo, Japan).

Western blotting

Cells were seeded and incubated separately in 55-cm^2 flasks under normoxia and under hypoxia for 3 h, 6 h, 12 h, 24 h. After incubation, the cells were rinsed 3 times with cold phosphate-buffered saline (PBS) for 5 min, then scraped into 200 μl of lysis buffer (RIPA: PMSF=100: 1), and protein concentrations were determined by use of the BCA assay kit (Beyotime Institute of Biotechnology, Shanghai, China) in accordance with the manufacturer's instructions, and bovine serum albumin was used as a standard. We collected 30 μg of proteins and electrophoresed it using 8–12% SDS-PAGE gels at 100 V, then it transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, MA, USA). The membranes were blocked in a

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Table 1. Primer sequences used for real-time quantitative PCR.

Genes	Forward (5'-3')	Reverse (5'-3')
HIF-1α	CCAGATTCAAGATCAGCCAGCA	GGAAGAGGGAAAGGACAGCAT
BNIP3	TCTGGACGAAGCAGCTCCAA	CCAAAGCTGTGGGTGTCTATTTCA
β-actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG

5% non-fat milk-TBST solution for at least 60 min while shaking. Then, we rinsed the membranes 6 times for 5 min each time with TBS-0.05% Tween-20(TBST) at room temperature. Subsequently, the membranes were incubated with the rabbit anti-HIF-1α antibody (1: 500 dilution, Abcam), rabbit anti-BNIP3 antibody (1: 1000 dilution, Abcam), and rabbit anti-Beclin 1 antibody (1: 1000 dilution, Abcam) overnight at 4°C with shaking. To ensure equal protein loading, β-actin (1: 1000 dilution, ZSJB-BIO, China) was detected on the same membrane and used as a loading control. The blots were incubated with horseradish peroxidase-conjugated anti-rabbit antibody (1: 5000 dilution) for 60 min and visualized with the SuperSignal West Femto maximum sensitivity substrate (Amersham Imager 600, GE, Germany). The band density was calculated with ImageJ software packages.

Transmission electron microscopy (TEM)

MDSCs were seeded and incubated separately in 55-cm^2 flasks under normoxia or hypoxia for 24 h. Then, the cells were fixed with 2% glutaraldehyde (Beijing Chemical Industry Group, Co., Ltd., Beijing, China) in 0.1 M phosphate-buffered saline (PBS) pH 7.4 for 12 h at 4°C and rinsed twice for 30 min each time in 0.1 M PBS, postfixed with 1% osmium tetroxide (Beijing Chemical Industry Group, Co., Ltd.) dissolved in 0.1 M cacodylate buffer (Beijing Chemical Industry Group, Co., Ltd.) for 2 h at room temperature in the dark and dehydrated in an ascending gradual series (30–90%) of ethanol (Beijing Chemical Industry Group, Co., Ltd.). Specimens were embedded in Epon (Beijing Chemical Industry Group, Co., Ltd.). After pure fresh resin embedment and polymerization at 60°C, we made 50–70 nm ultrathin sections with a UC7 microtome (Leica Microsystems, Wetzlar, Germany). All thin sections were observed by TEM (JEM-1200, JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

Reverse transcription-quantitative polymerase chain reaction (RT-PCR)

After incubation under normoxia or hypoxia for 3, 6, 12, and 24 h, the cells are detached. We added TRIZOL (TaKaRa Co., Ltd., Dalian) and total RNA was harvested using the Total RNAgent extraction kit (TaKaRa Co., Ltd., Dalian, China) following the manufacture's protocol. The mRNAs were analyzed by RT-PCR. The mRNA contained in 1 μg total RNA was reverse transcribed using the PrimeScript™ RT reagent kit (Takara Biotechnology, Co., Ltd.) with gDNA Eraser (Takara Biotechnology, Co., Ltd.) and oligodT primers (Table 1) according to the manufacturer's instructions. Real-time PCR was conducted with a Roche Light Cycler 480 device (Roche Applied Science, Germany) in a total volume of 20 μl reacting system. The primer nucleotide sequences for PCR are presented in Table 1. The PCR reactions included an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and dissociation at 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s. β-actin was used as the reference gene for normalization and mRNA expression level was quantified using the threshold cycle method.

Statistical analysis

All data are expressed as the mean ± standard error of the mean of at least 3 independent experiments. Differences in measured variables were assessed with one-way analysis of variance (ANOVA). Differences were considered significant at $P<0.05$.

Results

Isolation and identification of the MDSCs

The MDSCs were isolated by a modified pre-plate technique as described in Materials and Methods. MDSCs are rare cell within muscle, at a ratio of approximately 1 per 100 000 cells. Although many attempts have been made to better identify MDSCs, no standard markers or proteins for MDSCs exist. However, increasing evidence demonstrates that MDSCs have a high expression of stem cell antigen-1 (Sca-1), cluster of differentiation 34 (CD34), Bcl-1, and desmin, while they are negative for CD45 [10]. In this study, the immunofluorescence staining was performed to identify the MDSCs. Consistent with previous studies, CD34, Sca-1, and desmin were expressed positively in 80–90% and CD45 was negatively expressed in the MDSCs we isolated (Figure 1). Therefore, the cells were qualified for use in subsequent experiments.

Hypoxia enhanced autophagy in MDSCs

To investigate whether autophagy is involved under hypoxia stress, we first analyzed the levels of 2 important autophagy proteins: LC3B-II and Beclin-1. Under normoxia, LC3B protein

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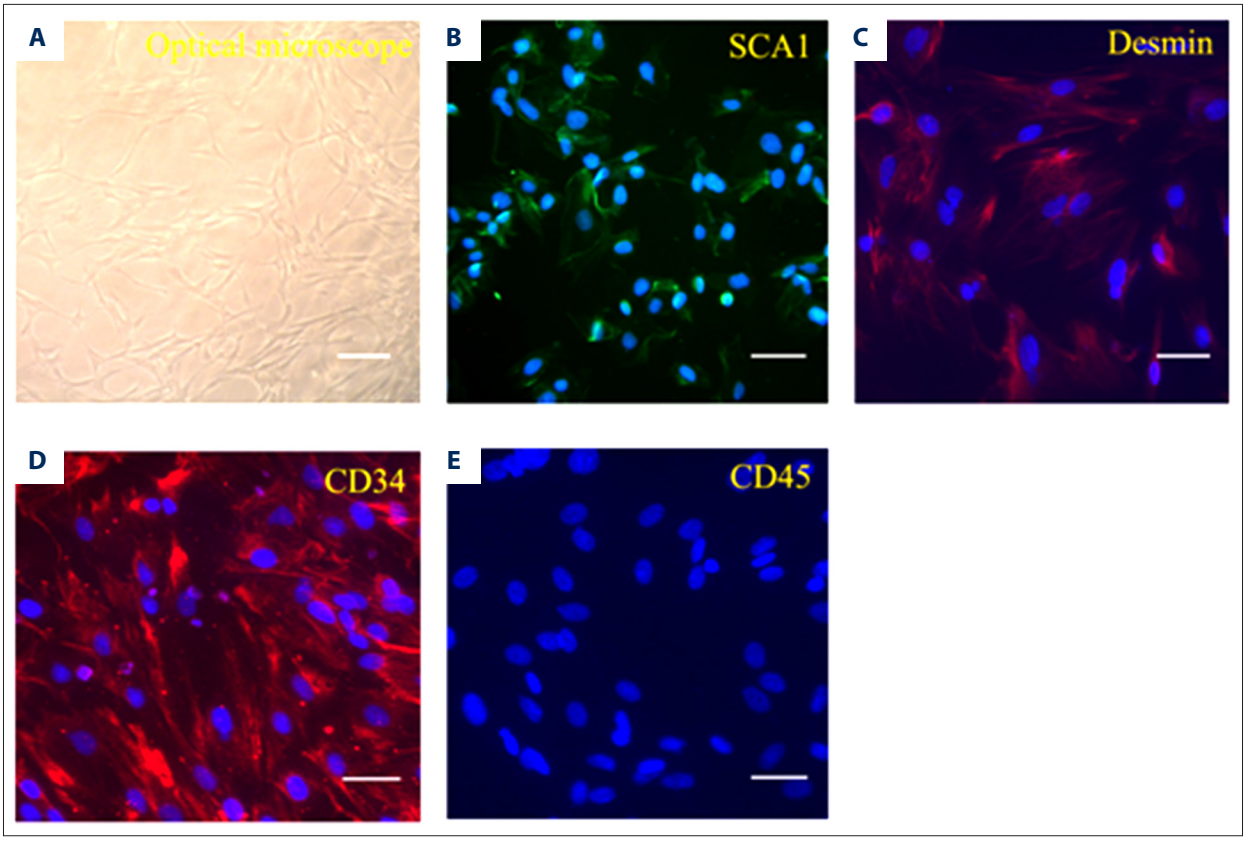

Figure 1. Identification of MDSCs derived from rat genioglossus muscle. (**A**) Optical microscope image of representative morphological characterization before differentiation at passage 3 (×20). (**B**) Immunofluorescence staining of Sca-1 (×20). (**C**) Immunofluorescence staining of Desmin (×20). (**D**) Immunofluorescence staining of CD34 (×20). (**E**) Immunofluorescence staining of CD45 (×20).

exists as LC3B-I in the cytosol, and when autophagy is activated, LC3B-I recruits to the autophagosome membrane and is converted to LC3B-II [11]. Beclin-1, which involved in the initiation phase of autophosome formation, is another marker used in assessment of autophagy levels [11]. Our results showed that the expressions of LC3B-II and Beclin-1 were increased in a time-depend manner in hypoxia-treated MDSCs compared with cells under normoxic conditions (Figure 2A–2C). These data suggest that hypoxia treatment increased autophagy levels in MDSCs.

To further confirm an increase in the autophagy levels induced by hypoxia, transmission electron microscopy (TEM) was used to explore morphological evidence of autophagy flux. The results showed that the number of double-membrane autophagosomes increased significantly when cells were incubated in hypoxia conditions for 12 and 24 h. Cells in normoxic conditions showed normal mitochondria and endoplasmic reticulum (Figure 2D), while cells exposed to hypoxia demonstrated deposition of autophagosomes, with cytoplasmic organelles and other vesicles encapsulated in vacuoles (Figure 2E, 2F). Taken together, the results show that autophagy was activated in MDSCs under hypoxia stress.

HIF-1α/BNIP3 signal pathway was involved in hypoxia-induced autophagy in MDSCs

As we showed above that hypoxia treatment increased autophagy levels in MDSCs, we next explore the mechanisms involved in this process. Previous studies have shown that hypoxia upregulates the expression of HIF-1α in many different cell lines, and the HIF-1α/BNIP3 signal pathway has been found to play an essential role in hypoxia-induced autophagy [12]. Thus, we investigated whether HIF-1α and BNIP3 are involved in hypoxia-induced autophagy in MDSCs. MDSCs were incubated under the normoxic and hypoxic conditions for 3 h, 6 h, 12 h, and 24 h, and the total RNA and proteins were harvested to perform RT-PCR and Western blot analysis. Results showed that the mRNA expression of HIF-1α increased significantly at 3 h under hypoxic conditions. Moreover, HIF-1α protein also started to increase rapidly within 12 h of exposure to hypoxia (Figure 3). Similar results were produced in the BNIP3 study. In conclusion, these data revealed that the effect of hypoxia in autophagy induction were at least partly due to upregulation of the HIF-1α/BNIP3 signal pathway.

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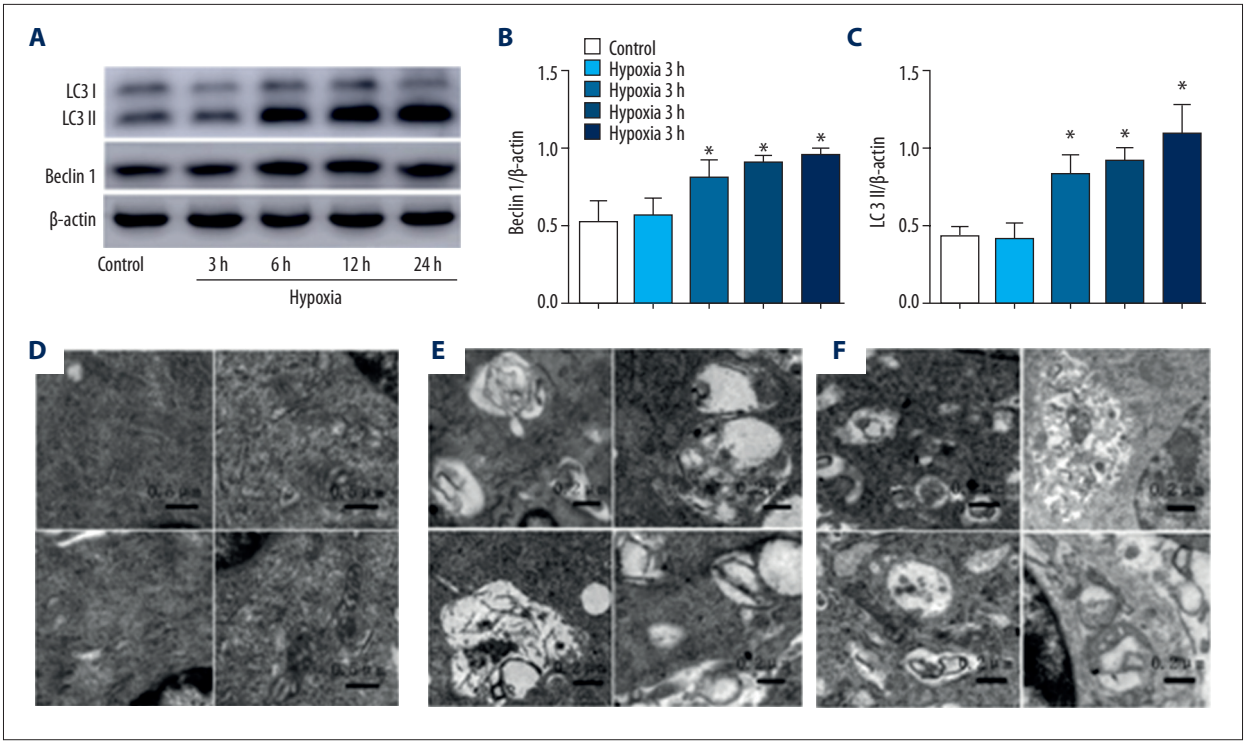

Figure 2. Hypoxia induces autophagy in MDSCs. MDSCs were incubated separately in 55-cm^2 flasks under the normoxia or hypoxia for indicated times (3, 6, 12, and 24 h). (**A**) Representative Western blot analysis of LC3 and Beclin 1. (**B**) Quantification of Beclin 1 and (**C**) LC3 expression relative to the β-actin level in different groups. * p<0.05, compared to control group, n=3. (**D**) Numerous autophagosomes containing cytoplasmic organelles and other vesicles under hypoxic conditions for 12 h (**E**) and 24 h (**F**) hypoxia by TEM. * *p*<0.05, compared to control group, n=3.

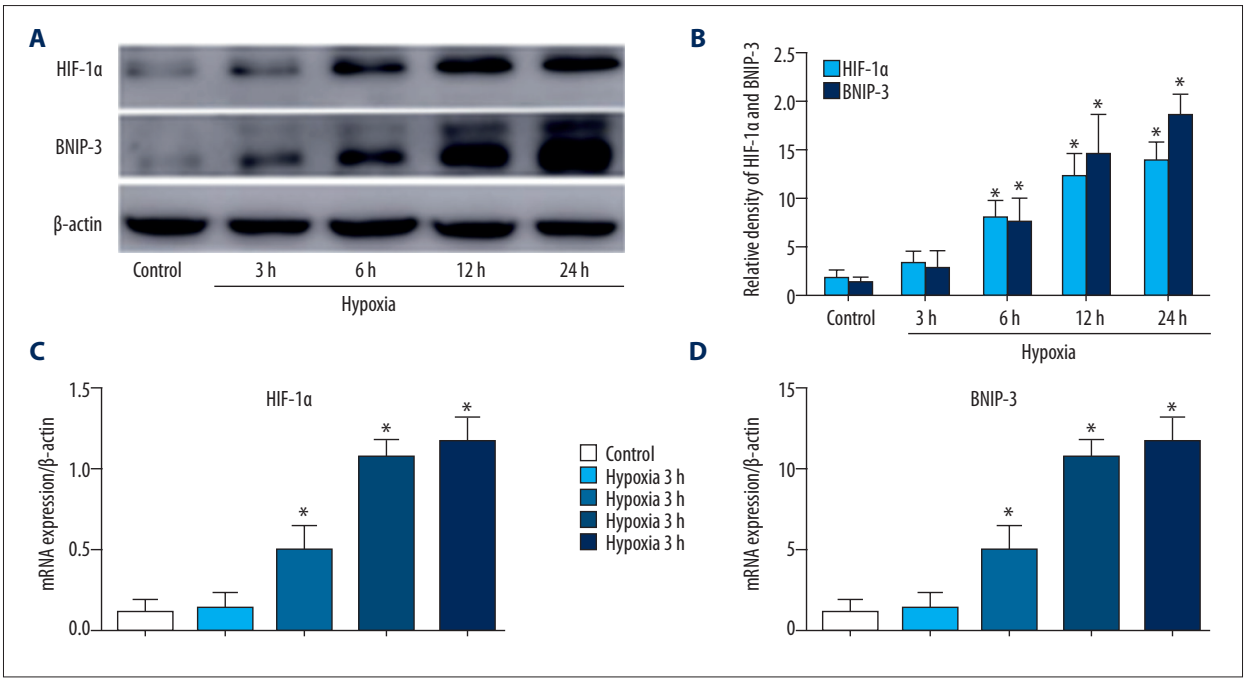

Figure 3. The protective response of HIF-1α under hypoxic conditions in MDSCs. The protein (**A, B**) and mRNA (**C, D**) expressions of HIF-1α and BNIP3 were detected by Western blot and RT-PCR, respectively. * P<0.05, compared to control group, n=3.

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Discussion

Obstructive sleep apnea (OSA) is caused by the dysfunction of upper airway dilator muscles, with genioglossus (GG) being the most important one [1,2]. Upper airway collapse leads to chronic intermittent hypoxia and, subsequently, sustained hypoxia. Previous studies have found that sustained hypoxia in people with OSA impaired the upper airway muscle activity [3], especially for genioglossus cells. In turn, depressed GG activity was found to result in hypoxic ventilatory depression (HVD), which arises in the late phase of the biphasic ventilatory response during sustained hypoxia [4]. Thus, research on the interaction between hypoxia and genioglossus is of great importance in understanding the pathogenesis of OSA.

Autophagy is an evolutionarily and highly conserved dynamic lysosomal degradation process for degradation of misfolded protein and damaged organelles [6]. Autophagy has emerged as an important regulator under hypoxic conditions. Zhang [13] proposed that hypoxia-induced autophagy was beneficial to cell survival by eliminating the dysfunctional mitochondria and preventing an increase in the level of reactive oxygen species, but Azad [14] demonstrated that prolonged exposure to hypoxia could result in autophagic cell death. It has been showed that sustained hypoxia reduced the activity of GG in OSA in a clinical trial [4]. However, the role of autophagy in sustained hypoxia-induced GG damage has not been reported. As GG MDSCs are earlier progenitors found within skeletal muscle and possess a higher regeneration capacity and a broader range of multilineage capabilities, we explored the level of autophagy activity of GG MDSCs during sustained hypoxia. Our results showed that sustained hypoxia for 6 h, 12 h, and 24 h promoted autophagy in GG MDSCs, revealing that autophagy plays an important role in genioglossus MDSCs against sustained hypoxia stress.

As a key transcription factor in rapid adaptation to hypoxia, hypoxia inducible factor-1 alpha (HIF-1α) plays a crucial role in OSA [15]. HIF-1α regulates the transcription of many genes in response to hypoxia and controls a series of molecular mechanisms related to energy homeostasis [16]. In our previous study, HIF-1α was found to be widely expressed in the genioglossus muscle under hypoxia condition [17], and recent studies revealed that the HIF-1α/BNIP3 signaling pathway is closely related to autophagy reduction in cancer cells in hypoxia [18]. Previous studies revealed that Bcl-2 interacts with Beclin 1, the mammalian orthologue to yeast ATG6, and inhibits its autophagic activity by blocking its interaction with Vps34 [19]. However, several recent studies have substantiated that HIF-1α forms

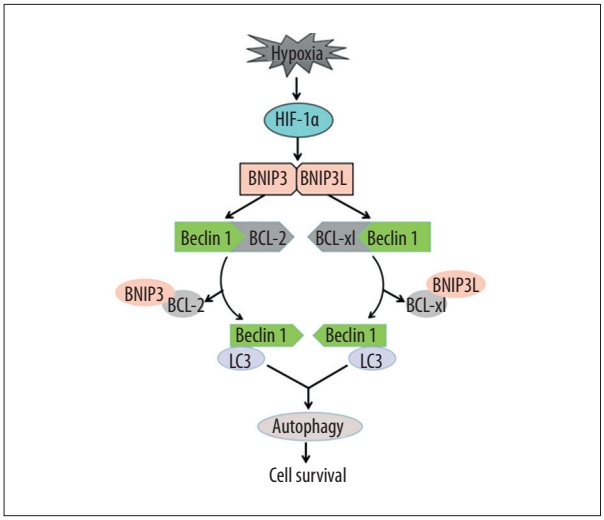

Figure 4. Schematic diagram. The molecular mechanisms by which hypoxia affects autophagy in GG MDSCs.

an HIF-1 heterodimer, and upregulates BNIP3 to disrupt the Beclin 1/Bcl-2 complex, then subsequently releases Beclin 1 to induce autophagy under hypoxic conditions [18]. Furthermore, it was reported that the HIF-1α/BNIP3 pathway plays a crucial role in hypoxia-induced autophagy in other cells, such as cardiomyocytes, synoviocytes, and neuronal cells [18]. In the present study, compared with normoxia, HIF-1α expression increased dramatically with the extended exposure to hypoxia; this corresponds with upregulation of BNIP3 to release Beclin 1, and autophagy was induced.

Conclusions

The present study revealed that sustained hypoxia activated autophagy in GG MDSCs, which played a protective role against hypoxia stress. The HIF-1α/BNIP3 pathway may be critical in modulating the sustained hypoxia-induced autophagy in GG MDSCs (Figure 4). These findings give insight into the critical role of autophagy in the pathogenesis of OSA.

Conflict of interest

None.

Acknowledgment

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