Overexpression of transcription factor EB regulates mitochondrial autophagy to protect lipopolysaccharide-induced acute lung injury

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Abstract

Background: Acute lung injury (ALI) is characterized by an acute inflammatory process, and oxidative stress in the lung tissue leads to a lack of effective therapeutics. This study aimed to identify whether the overexpression of transcription factor EB (TFEB) regulates mitophagy to protect against lipopolysaccharide (LPS)-induced ALI.

Methods: We detected the expression of inflammatory factors, cytochrome c (Cyt.c) and nicotinamide adenine dinucleotide phosphate (NADPH), and autophagy-related proteins and observed the changes in lung histopathology induced by ALI in rats and the changes in the cell ultrastructure of primary alveolar type II epithelial cells induced by changing the expression of TFEB in the context of ALI.

Results: The overexpression of TFEB could reduce the expression of proinflammatory factors, such as IL-1 and IL-6, and increase the expression of anti-inflammatory factors, such as IL-10, both in vitro and in vivo. In addition, the overexpression of TFEB could reduce the Cyt.c and NADPH levels both in vivo and in vitro. The overexpression of TFEB could upregulate the expression of autophagy-related proteins, such as lysosomal-associated membrane protein 1 (LAMP1), microtubule-associated protein light chain 3B (LC3B), and Beclin both in vivo and in vitro, and promote mitochondrial autophagy. The overexpression of TFEB significantly improved the histopathologic changes induced by LPS-induced ALI in rats. However, low TFEB expression produced the opposite results.

Conclusion: TFEB overexpression can decrease inflammation and mitochondrial damage in the lung tissue and alveolar epithelial cells through regulating mitochondrial autophagy to protect against LPS-induced ALI. Therefore, TFEB is likely a potential therapeutic target in LPS-induced ALI.

Keywords: Transcription factor EB; Mitochondrial autophagy; Acute lung injury; Inflammation; Mitochondrial damage

Introduction

Acute lung injury (ALI) is a life-threatening disease with high mortality and morbidity, which is characterized by an acute inflammatory process and oxidative stress in the pulmonary parenchyma and interstitial tissue, alveolar epithelial cell dysfunction, pulmonary oxidative damage, and edema. ALI will progress to Acute Respiratory Distress Syndrome (ARDS) if it is not effectively controlled in the early stage. ARDS is a form of respiratory failure that exhibits higher mortality. Thus, how to effectively treat ALI in the early stage is an important problem.

Autophagy plays a complex role in human diseases, as it is both protective and injurious. Autophagy can protect against ALI through restricting injury to the lung microvascular barrier. Mitochondrial damage can release vast amounts of oxidases that aggravate lung tissue injury in sepsis-induced diseases, such as lipopolysaccharide (LPS)-induced ALI and renal injury. Mitochondrial autophagy is an important way to remove damaged mitochondria and a mechanism for self-preservation. It is not known whether the upregulation of mitochondrial autophagy can protect against ALI.

Transcription factor EB (TFEB) is an important transcription factor in autophagy, which can directly regulate the expression of autophagy-associated proteins. Therefore, we hypothesized that TFEB can regulate mitochondrial autophagy to protect against LPS-induced ALI. In the study, we demonstrated that TFEB regulates inflammatory factors and mitochondrial damage in ALI by regulating mitochondrial autophagy and the expression of autophagy-associated proteins.
These findings suggest that TFEB might be a potential therapeutic target in ALI.

**Methods**

**Animal and materials**

Adult male Sprague-Dawley rats (180–220 g) were obtained from the Animal Center of the Air Force Military Medical University (Xi’an, China). Animal experiments were approved by the Animal Care and Use Committee of the Air Force Military Medical University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Publication No. 85-23, revised 1985). Anti-lysosomal-associated membrane protein 1 (LAMP1, ab24170), anti-Beclin (ab62557), anti-microtubule-associated protein light chain 3B (LC3B, ab63817), and anti-GAPDH (ab37168) antibodies were purchased from Abcam (Cambridge, UK). An anti-TFEB antibody (13372-1-AP) was purchased from Proteintech (Rosemont, IL, USA). Anti-SP-A (sc-13977) was purchased from Santa Cruz (Dallas, TX, USA). Cytochrome c (Cyt.c) assay (ELISA) kits were purchased from Sigma Chemical Company (St. Louis, MO, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) assay kits were purchased from Sigma Chemical Company (St. Louis, MO, USA). Cytochrome c (Cyt.c) assay (ELISA) kits were purchased from Solarbio Life Sciences (Beijing, China).

**Alveolar type II epithelial cell isolation and culture**

Primary alveolar type II epithelial cells were isolated from Sprague-Dawley rats as described in a previous article.[10] The isolated cells are resuspended to 10^6 cells/mL in Dulbecco’s modified Eagle’s medium (DMEM) with 10% bovine serum (FBS), then cultured for 12 h in noncoated cell culture plate in a humidified 5% CO2 incubator at 37°C to remove residual mesenchymal cells attached to the plate. The unattached cells (mainly alveolar type II epithelial cells) are gently collected and centrifuged at 300 x g for 10 min at 4°C. The pellet is resuspended in DMEM with 10% FBS and cultured on a coated plate in 5% CO2–95% air at 37°C. The cells were identiied by immunofluorescence staining. Steps were as follows. Slides of cells were fixed with 4% formaldehyde (15 min). After washed with PBS, cells were incubated with 10% normal goat serum and 5% Bull Serum Albumin (1 h), and then incubated with anti-SP-A overnight at 4°C in moist box. Cells were incubated with 594-Goat-anti-Rat for 30 min at indoor temperature. After washed with PBS, diisopropylamonnium was added (5 min) and cells were subsequently examined under a fluorescence microscope.

**Plasmids and lentivirus preparation**

The TFEB gene product was cloned into the pCDH-CMV-MCS-EF1a-copGFP vector. A TFEB shRNA sequence was also cloned into the pCDH-CMV-MCS-EF1a-copGFP vector. The pLenti/EGFP transgene vector and the packaging plasmids pLP1 and pLP2 as well as the pLP and VSVG plasmids encoding the viral proteins pCDH-CMV-TFEB-EF1-copGFP and pCDH-CMV-shTFEB-copGFP, respectively, were prepared and transfected as previously described.[11]

**Cell transfection and treatment**

Primary alveolar type II epithelial cells were cultured in six-well culture dishes. There were four groups: the control group, LPS group, LPS + TFEB group (LPS + TFEB lentivirus), and LPS + shTFEB group (LPS + shTFEB lentivirus). After being challenged with 10 μg/mL LPS for 24 h, the cells were incubated in serum-free medium for 12 h. Then, the cells were infected with 20 μL TFEB lentivirus (virus titer: 7.5 x 10^8) or shTFEB lentivirus (virus titer: 3.4 x 10^8) for 48 h in DMEM supplemented with 10% FBS in 5% CO2–95% air at 37°C. Then, the cell supernatant and cell protein of each group was collected.

**Cell ultrastructure**

After challenge and infection, the primary alveolar type II epithelial cells from each group were collected into the AGAR empty slot in a centrifuge tube. Then, 2.5% glutaralddehyde was used to fix the fluid cells. Semithin (1-mm-thick) sections were cut from the AGAR empty slot, which included the fixed cells. The fixed cell sections were postfixed for 1 h in 1% osmium tetroxide and dehydrated in alcohol. The cells were examined under a TEM-100CX electron microscope (Japan Electron Optical Laboratory, Tokyo, Japan).

**Animal model and grouping**

There were four groups of rats: the control group, LPS group (LPS-induced ALI group), LPS + TFEB group (LPS-induced ALI + TFEB lentivirus), and LPS + shTFEB group (LPS-induced ALI + shTFEB lentivirus). There were five rats in each group. We established an ALI model through a tail vein injection (LPS, 10 mg/kg). The same volume of normal saline was injected via the tail vein in the control group. After 6 h the ALI model was established, rats were injected with 3% pentobarbital sodium 0.3 mL intraperitoneally with mild anesthesia. Then lentivirus suspension drops (5.0 x 10^8 transduction units per rat) were instilled into the rat lungs through the trachea intermittently. The control group was inhaled with the same volume of 0.9% normal saline. The rats were sacrificed by aortic transection at 48 h after lentivirus instillation. We demonstrated transfection efficiency via observing the expression of GFP in lung tissue under fluorescence microscope.

**Preparation of the BALF**

Forty-eight hours after the lentivirus was instilled, the rats were anesthetized. The left lung was lavaged with 1-mL ice-cold phosphate-buffered saline five times in each group. We recovered 90% of the bronchoalveolar lavage fluid (BALF). The collected BALF was centrifuged at 520 x g for 20 min at 4°C, and the supernatant was used for subsequent studies.

**Western blotting**

The lung tissue samples from each group were collected after the rats were perfused with pH 7.4 phosphate-buffered saline.
saline to remove the blood cells from the pulmonary circulation. Total protein extracted from the primary alveolar type II epithelial cells and lung tissue was prepared according to the instructions of a Total Protein Extraction Kit. Protein concentrations were determined through the BCA method. Protein samples were separated on a denaturing 8% or 6% Sodium Dodecyl Sulfonate-polyacrylamide gel and transferred to a nitrocellulose membrane, which was incubated with rabbit monoclonal antibodies overnight. The antibody concentrations were 1:1000 anti-LAMP1 antibody, 1:1000 anti-LC3B antibody, 1:500 anti-TFEB antibody, 1:1000 anti-Beclin antibody, and 1:2500 anti-GAPDH antibody. The secondary antibody (anti-rabbit, 1:5000) was incubated for 1.5 h. Detection was performed using an enhanced chemiluminescence system (Amersham, Arlington Heights, IL, USA).

**ELISA**

The levels of IL-1, IL-6, IL-10, Cyt.c, and NADPH in the cell supernatant and BALF were determined by using commercially available ELISA kits according to the manufacturer’s instructions.

**Histopathological sampling and scoring**

At 48 h after lentivirus instillation, all groups of rats were sacrificed by aortic transection. The lung tissue was removed and fixed with 4% paraformaldehyde for 24 h. The lower lobe of each right lung was embedded in paraffin and cut into 5 μm sections. Hematoxylin and eosin staining was performed according to a standard protocol.

For scoring, five samples were cut into two sections in each group so that the total number of sections per group was 10. The sections were examined by two independent pathologists who were blinded to the group assignments. Each section was evaluated for the lung injury score described in a previous standard assessment, which ranged from 0 to 4 based on edema, neutrophil infiltration, hemorrhage, bronchiole epithelial desquamation, and hyaline membrane formation. The score ranged from 0 to 4 and represented the ALI severity: 0 for no injury, 1 for modest injury, 2 for intermediate injury, 3 for widespread injury, and 4 for the most prominent injury.

**Statistical analysis**

Data are expressed as the mean ± standard error. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Dunnett test for multiple comparisons. A statistically significant difference was defined as $P < 0.05$.

**Results**

**Effects of TFEB on inflammatory factors in LPS-induced ALI**

The results showed that compared with the corresponding LPS groups, the LPS + TFEB groups showed reduced expression of IL-1 (cell supernatant level reduced from 118.47 to 0.49, $P < 0.05$, $n = 3$; BALF level reduced from 43.69 to 0.65, $P < 0.05$, $n = 5$) and IL-6 (cell supernatant level reduced from 25.75 to 4.66, $P < 0.05$, $n = 3$; BALF level reduced from 18.94 to 3.10, $P < 0.05$, $n = 5$) and increased expression of IL-10 (cell supernatant level increased from 9.76 to 32.79, $P < 0.05$, $n = 3$; BALF level increased from 1.05 to 8.71, $P < 0.05$, $n = 5$).

![Figure 1](image-url)

**Figure 1:** Effects of TFEB on the inflammatory factors in the cell supernatant (A–C) and in the BALF (D–F). The overexpression of TFEB could reduce the expression of IL-1 and IL-6 and increase the expression of IL-10. The knockdown of TFEB expression could increase the expression of IL-1 and IL-6 and reduce the expression of IL-10. Data are presented as the mean ± SEM ($n = 3$ for the cell supernatant, and $n = 5$ for the BALF). $^*\quad P < 0.05$ vs. control group; $^\dagger\quad P < 0.05$ vs. LPS group. BALF: Bronchoalveolar lavage fluid; LPS: Lipopolysaccharide; TFEB: Transcription factor EB.
level increased from 9.23 to 11.41, \( P < 0.05, n = 5 \). However, compared with the corresponding LPS groups, the LPS + shTFEB groups exhibited increased expression of IL-1 (cell supernatant level increased from 118.47 to 606.41, \( P < 0.05, n = 3 \); BALF level increased from 27.66 to 323.70, \( P < 0.05, n = 5 \)) and IL-6 (cell supernatant level increased from 25.75 to 46.59, \( P < 0.05, n = 3 \); BALF level increased from 18.94 to 42.28, \( P < 0.05, n = 5 \)) and reduced expression of IL-10 (cell supernatant level decreased from 9.76 to 6.03, \( P < 0.05, n = 3 \); BALF level decreased from 9.23 to 7.22, \( P < 0.05, n = 5 \)) [Figure 1].

**Effects of TFEB on mitochondrial damage in LPS-induced ALI**

As shown in Figure 2, compared with the corresponding LPS groups, the LPS + TFEB groups exhibited decreased levels of Cyt.c (cell supernatant level decreased from 19.85 to 8.15, \( P < 0.05, n = 3 \); BALF level decreased from 21.41 to 5.79, \( P < 0.05, n = 5 \)) and NADPH (cell supernatant level decreased from 129.00 to 57.78, \( P < 0.05, n = 3 \); BALF level decreased from 138.11 to 74.78, \( P < 0.05, n = 5 \)), whereas the LPS + shTFEB groups displayed increased levels of Cyt.c (cell supernatant level increased from 19.85 to 37.05, \( P < 0.05, n = 3 \); BALF level increased from 21.41 to 40.40, \( P < 0.05, n = 5 \)) and NADPH (cell supernatant level increased from 129.00 to 165.89, \( P < 0.05, n = 3 \); BALF level increased from 138.11 to 165.56, \( P < 0.05, n = 5 \)).

**Effects of TFEB on mitochondrial autophagy-related proteins in LPS-induced ALI**

We measured the expression of mitochondrial autophagy-related proteins, such as LAMP1, Beclin, and LC3B. The results showed that the protein expression of LAMP1, Beclin, and LC3B was obviously increased in the LPS + TFEB groups but decreased in the LPS + shTFEB groups compared with the corresponding LPS groups in vitro and in vivo [Figure 3].

**Effects of TFEB on histopathology in LPS-induced ALI**

Histopathologic staining showed that compared with control, LPS induced lung edema, inflammatory cell infiltration in the pulmonary mesenchyme and alveoli, hemorrhage, bronchiole epithelia desquamation, and hyaline membrane appearance [Figure 4B]. Compared with the LPS group, the LPS + TFEB groups showed obvious improvements in lung edema, inflammatory cell infiltration,
and hemorrhage [Figure 4C]. In the LPS + shTFEB group, the inflammatory cell infiltration was more serious than that in the LPS group [Figure 4D]. The assigned lung injury scores of every group are shown in Table 1.

**Effects of TFEB on cell ultrastructure**

To further clarify the effects of TFEB on mitochondrial autophagy, we observed the cell ultrastructure of primary alveolar type II epithelial cells using electron microscopy. In the control group, the cells showed normal ultrastructure. In the LPS group, the autophagosome reactivity was stronger than that of the control group. There were more autophagosomes in the LPS + TFB group than those in the LPS group. Compared with the LPS group, the LPS + shTFEB group exhibited decreased autophagosome accumulation [Figure 5].

**Discussion**

In this study, we demonstrated that TFEB regulated mitochondrial autophagy to protect against LPS-induced...
The overexpression of TFEB could reduce the expression of proinflammatory factors, such as IL-1 and IL-6, and increase the expression of anti-inflammatory factors, such as IL-10, both in vitro and in vivo. The overexpression of TFEB could upregulate the expression of autophagy-related proteins, such as LAMP1, LC3B, and Beclin; reduce the expression of Cyt.c and NADPH both in vitro and in vivo; and significantly increase the accumulation of autophagosomes in alveolar type II epithelial cells. The overexpression of TFEB significantly improved the histopathologic changes associated with LPS-induced ALI in rats. However, knocking down the expression of TFEB had opposite results.

ALI is characterized by noncardiogenic pulmonary edema with alveolar epithelial cell and pulmonary mesenchyme inflammation and oxidative stress. The main treatment strategies for ALI include ventilation, anti-inflammatory, antioxidant, and mesenchymal stem cell therapies; however, none of these treatment strategies is the most effective. Therefore, in the present study, we focused on a potentially effective treatment for ALI. The mitochondria are the only organelles containing DNA other than the nucleus so they are very sensitive to oxidative stress and liable to be damaged. When the mitochondria are damaged in ALI, vast amounts of oxidases are released; excessive oxidase release then leads to more serious oxidative stress and mitochondrial damage. When the mitochondria are damaged, mitochondrial respiratory chain products, such as Cyt.c and NADPH, are released. Therefore, the increases in the levels of Cyt.c and NADPH are signs of mitochondrial damage. Mitophagy is the mechanism of mitochondrial damage self-repair. Upregulating mitophagy decreases the levels of mitochondrial respiratory chain products, such as Cyt.c and NADPH. Therefore, Cyt.c and NADPH are the biomarkers of mitophagy. In this study, we found that the Cyt.c and NADPH levels were

| Group       | Edema     | Inflammatory cell infiltration | Hemorrhage | Bronchiole epithelial desquamation |
|-------------|-----------|---------------------------------|------------|----------------------------------|
| Control     | 0.20 ± 0.13 | 0.20 ± 0.17                      | 0.30 ± 0.19 | 0.40 ± 0.26                      |
| LPS         | 3.30 ± 0.22* | 3.40 ± 0.14*                      | 3.10 ± 0.48* | 2.90 ± 0.31*                      |
| LPS + TFEB  | 2.10 ± 0.25† | 1.80 ± 0.28†                      | 2.20 ± 0.31† | 1.10 ± 0.35†                      |
| LPS + shTFEB| 2.80 ± 0.45† | 3.80 ± 0.12†                      | 2.30 ± 0.29† | 2.80 ± 0.25†                      |

Data were represented as mean ± standard error. *P < 0.05 vs. control group; †P < 0.05 vs. LPS group, n = 10. ALI: Acute lung injury; LPS: Lipopolysaccharide; TFEB: Transcription factor EB.
significantly increased in the LPS group and significantly decreased in the LPS + TFEB group.

Some studies found that upregulated autophagy can decrease mitochondrial damage and lung inflammation.[27] TFEB was the first identified member of the MiTF/TFE family, which include of TFEB, TFE3, MITF, and TFEC,[28] and plays a pivotal role in the regulation of lysosomal biogenesis and autophagy.[29] However, it is not known whether upregulating TFEB expression will improve mitochondrial autophagy and protect against ALI. In our study, we observed that the overexpression of TFEB could promote autophagy in damaged mitochondria under an electron microscope. We also found that the overexpression of TFEB could upregulate the expression of autophagy-related proteins and decrease inflammation and Cyt.c and NADPH expression, whereas knocking down the expression of TFEB exerted opposite effects. Thus, TFEB likely regulates mitochondrial damage by regulating autophagy-related proteins.

In conclusion, upregulated mitochondrial autophagy can decrease inflammation and mitochondrial damage in LPS-induced ALI. The overexpression of TFEB can upregulate mitochondrial autophagy through upregulating autophagy-related protein expression to protect against LPS-induced ALI. Thus, TFEB might be a potential therapeutic target in LPS-induced ALI.

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Conflicts of interest
None.

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