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# 4-PBA inhibits LPS-induced inflammation through regulating ER stress and autophagy in acute lung injury models

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## HIGHLIGHTS

- 4-PBA protects LPS-induced acute lung injury and inflammation in mouse model.
- 4-PBA decreases the levels of ER stress and autophagy induced by LPS *in vivo* and *in vitro*.
- Inhibition of autophagy by 3-MA aggravates cell injury induced by LPS, ER stress-associated autophagy may play a protective effect in LPS-induced lung injury.

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## ABSTRACT

Acute lung injury (ALI) is a common clinical disorder that causes substantial health problems worldwide. An excessive inflammatory response is the central feature of ALI, but the mechanism is still unclear, especially the role of endoplasmic-reticulum (ER) stress and autophagy. To identify the cellular mechanism of lung inflammation during lipopolysaccharide (LPS)-induced mouse model of ALI, we investigated the influence of classic ER stress inhibitor 4-phenyl butyric acid (4-PBA) on ER stress and autophagy, which partially affect the activation of inflammation, both in LPS-induced ALI mouse model and human alveolar epithelial cell model. We demonstrated that 4-PBA, which further prevented the activation of the NF-κB pathway, decreased the release of the pro-inflammatory mediators IL-1β, TNF-α and IL-6, significantly inhibited LPS-activated ER stress. Moreover, it was found that autophagy was also decreased by the treatment of 4-PBA, which may play a protective role in ALI models through the classical AKT/mTOR signaling pathway. Inhibition of autophagy by 3-MA exacerbates cytotoxicity induced by LPS in A549 alveolar epithelial cells. Taken together, our study indicated that ER stress is a key promoter in the induction of inflammation by LPS, the protective effect of 4-PBA is related to the inhibition of ER stress and autophagy in LPS-induced ALI models. Furthermore, the role of autophagy that contributes to cell survival may depend on the activation of ER stress.



## 1. Introduction

Acute lung injury (ALI), along with the more severe condition acute respiratory distress syndrome (ARDS), is induced by a variety of insults, including endotoxins, acid aspiration, complement activation, and hyperoxia (Ather et al., 2010), and it is characterized by an excessive inflammatory response within the lungs and severely impaired gas exchange resulting from alveolar-capillary

*Abbreviations:* ALI, acute lung injury; LPS, lipopolysaccharide; ER stress, endoplasmic-reticulum stress; NF-κB, nuclear factor κB; IκB, inhibitor of κB.

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barrier disruption and pulmonary oedema (Matthay et al., 2012; Wheeler and Bernard, 2007). Inflammation is regarded as an important and ubiquitous feature of respiratory airway diseases (Sethi, 2010), it is widely accepted that dysregulation of inflammatory responses results in various aggravated lung diseases, such as ALI/ARDS, vascular diseases and even sepsis (Chung et al., 2009). Lipopolysaccharide (LPS) is able to activate signaling cascades for inflammatory mediators expression, such as tumor necrosis factor (TNF-α) and interleukin (IL)-6 (Rossol et al., 2011). The risks of inflammation are starkly demonstrated in a transgenic mouse that expressed a mutant version of the inhibitory protein IκB in which the activation of NF-κB in lung epithelial cells was sufficient to cause neutrophil recruitment, pulmonary oedema, arterial hypoxemia, and death in the absence of any infection or exogenous stimuli (Poynter et al., 2003), but the specific mechanism of this transcription factor remains to be further tested in ALI.

Endoplasmic reticulum (ER) stress is defined as the accumulation of unfolded or misfolded proteins in the ER and subsequently triggers the unfolded protein response (UPR), which are mediated by three transmembrane ER signaling proteins: pancreatic endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) (Hosoi and Ozawa, 2010). Engagement of UPR sensors triggers changes in downstream signaling such as X-box binding protein 1 (XBP1), CHOP, eukaryotic translation initiation factor 2 subunit alpha (eIF2α), which leads to the up-regulation of various UPR target genes to restore ER homeostasis (Ron and Walter, 2007; Wang et al., 2012). Recently, studies have demonstrated that ER stress is involved in LPS-induced lung inflammation *in vivo* and the expression of GRP78 and CHOP is up-regulated in LPS-stimulated airway epithelial cells *in vitro* (Kim et al., 2013). In LPS induced A549 alveolar epithelial type II cells injury model, LPS treatment caused the accumulation of p-PERK, p-eIF2α and nuclear ATF4, which triggered the UPR (Ye et al., 2015). However, the role of ER stress in LPS-induced lung inflammation is not fully understood.

Autophagy is a major catabolic process that delivers proteins, cytoplasmic components, and organelles to lysosomes for degradation and recycling (Gutierrez et al., 2004; Mizushima and Komatsu, 2011; Sir et al., 2010; Thurston et al., 2009; Wang et al., 2014). In general, autophagy could signify two possible functions. Autophagy represents an early adaptive mechanism of the tissue to the clearing of organelles or proteins for maintaining cell homeostasis. Moreover, excess autophagy results in autophagic cell death (Mizushima et al., 2008; Terman and Brunk, 2005). Evidence has been presented that indicates autophagy can promote cell survival or cell death depending on the cell type, the specific circumstances and different stimulus (Jin et al., 2012). Some studies have shown that increased autophagy reportedly plays an important role in ischaemia reperfusion-induced lung injury (Gao et al., 2013; Zhang et al., 2013). It is definitely confirmed that ER stress has emerged as a novel autophagy inducer and gained increased attention (Lee et al., 2012; Ullman et al., 2008; Yorimitsu and Klionsky, 2007; Zhang et al., 2016). It was demonstrated that autophagosomes could arise from other intracellular membrane structures, such as the ER, further evidences have suggested that the ER could contribute to autophagosome formation (Axe et al., 2008; Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009). Recently, LPS has been shown to activate ER stress and autophagy in A549 alveolar epithelial type II cells (Ye et al., 2015). However, there is no available information on the relationship between ER stress and autophagy in lung inflammation.

The inhibitor of ER stress, 4-phenyl butyric acid (4-PBA), is a low molecular weight compound that stabilizes protein conformation, improves the folding capacity of the ER and facilitates trafficking of mutant proteins to suppress ER stress (Kim et al., 2013; Yam et al., 2007). In this study, 4-PBA was added to suppress the ER stress signaling pathways, to evaluate protective effect of 4-PBA on LPS-induced acute lung injury mouse. In addition, we also employed a nonspecific inhibitor of PI3-kinase, 3-MA to further evaluate the link between ER stress and autophagy in LPS-stimulated A549 alveolar epithelial type II cells. Our data demonstrated that 4-PBA inhibited ER stress sensor proteins obviously and also decreased the level of autophagy, further inhibited inflammation. Moreover, the effect of 3-MA suggested that ER stress-mediated autophagy is likely to be an adaptive protective role in LPS-induced lung injury, the supplemental strategy to activate autophagy may contributes to the rescue of ALI.

## 2. Materials and methods

### 2.1. Animals and experimental protocol

Male ICR mice, 7–8 weeks of age and free of murine specific pathogens, were obtained from the Animal Center of the Chinese Academy of Science (Shanghai, China). Mice were randomized into groups consisting of control group, LPS (Sigma-Aldrich, L3129, LPS from E. coli 0127:B8) group, 4-PBA (Sigma Aldrich, USA) +LPS group and 4-PBA group. Mice were treated once by intratracheal instillation with 5 mg/kg of LPS in saline (or with saline as a control) under anesthesia using chloral hydrate (Matrix, Orchard Park, NY, USA). 4-PBA was administered 16–18 h (i.p., 10 mg/kg) before LPS treatment. Bronchoalveolar lavage fluid (BAL fluid) and lung tissue was performed at 6 h after intratracheal instillation of LPS. At the time of lavage, the mice (6 mice in each group) were narcotized using chloral hydrate. The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated. Precooled phosphate-buffered saline (PBS, Gibco-Invitrogen, Carlasbad, CA, USA) (0.8 ml each time) solution was slowly instilled into the lung and withdrawn. The collected solutions were pooled and then kept at 4 °C. A part of each pool was used for total cell counting. After centrifugation, the BAL fluid supernatants were stored at −80 °C until use. Cell pellets were resuspended with BSA for cell differentials or immunofluorescence staining. Total cell numbers were counted with a Nucleocounter (Chemometec., Gydevang, Denmark).

### 2.2. Histological study

Lung tissue for histological study was fixed in fresh 4% formaldehyde solution for 24 h and then dehydrated and embedded in paraffin, finally 5 μm thick sections were cut and stained with hematoxylineosin (H&E). The sections were counterstained with hematoxylin. The tissue sections were observed under a light microscope for the lung histopathology.

### 2.3. Enzyme-linked immunosorbent assay (ELISA)

The cytokine production of IL-1β, TNF-α and IL-6 in BAL fluid supernatants was quantified using a murine ELISA development kit (PEPROTECH, Rocky Hill, NJ) according to the manufacturer's recommendation.

### 2.4. Cell culture and drug treatment

The human type II lung epithelial A549 cell line was obtained from The Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were incubated in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) (GIBCO, USA) in a humidified atmosphere at 37 °C with 5% $CO_2$. 3-MA (5 mM) or 4-PBA (5 mM) dissolved in PBS were used to pretreat the cells for 2 h