Lipopolysaccharide Induces Autophagic Cell Death through the PERK-Dependent Branch of the Unfolded Protein Response in Human Alveolar Epithelial A549 Cells

Shaoying Li, Liang Guo, Pin Qian, Yunfeng Zhao, Ao Liu, Fuyun Ji, Liutong Chen, Xueling Wu, Guisheng Qian

Institute of Respiratory Disease, Xinqiao Hospital, Third Military Medical University, Chongqing, Department of Respiratory Medicine, Pudong New Area Gongli Hospital, Shanghai, Department of Respiratory Medicine, Kunming General Hospital of Chengdu Military Command, Kunming, Department of Respiratory Medicine, Renji Hospital, Shanghai Jiaotong University School of Medicine, Shanghai

Key Words
LPS • Autophagy • Endoplasmic reticulum stress • Unfolded protein response • PERK

Abstract
Background: Alveolar epithelial cell death plays a critical role in the pathogenesis of lipopolysaccharide (LPS)-induced acute lung injury. Increased autophagy has a dual effect on cell survival. However, it is not known whether autophagy promotes death or survival in human alveolar epithelial cells exposed to LPS. Methods: Genetic and pharmacological approaches were used to evaluate the effect of autophagy on A549 cell viability upon LPS exposure. The endoplasmic reticulum (ER) stress and unfolded protein response (UPR) pathways were examined with immunoblotting studies to further explore underlying mechanisms. Results: Treatment with LPS (50 μg/ml) led to autophagy activation and decreased cell viability in A549 cells. Blocking autophagy via short interfering RNA or inhibitor significantly decreased, whereas rapamycin increased, the LPS-induced effect on viability. ER stress was activated in LPS-stimulated A549 cells, and ER stress inhibitor reduced LPS-induced autophagy. LPS activated only the PERK pathway and had rarely effect on the ATF6 and IRE1 branches of the UPR in A549 cells. Moreover, the knockdown of PERK and ATF4 attenuated LPS-induced autophagy and promoted cell survival. Conclusion: In human alveolar epithelial A549 cells, LPS induces autophagic cell death that depends on the activation of the PERK branch of the UPR upon ER stress.

S. Li and L. Guo contributed equally to this work.

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Professor Guisheng Qian
and Dr. Xueling Wu
Institute of Respiratory Disease, Xinqiao Hospital, Third Military Medical University
183 Xinqiao Street, Chongqing, 40037 (China)
E-Mail guishengqian1220@163.com, E-Mail wuxueling76@126.com
Introduction

Alveolar epithelial cell damage has been recognized as a prominent feature of acute lung injury/acute respiratory distress syndrome (ALI/ARDS) and is known to contribute to the pathogenesis of this syndrome [1, 2]. The degree of alveolar epithelial injury has been considered to represent an important prognostic marker of ARDS [3]. Lipopolysaccharide (LPS), a component of the outer cell walls of Gram-negative bacteria, has been considered one of the important factors leading to ALI [4]. Previous studies suggested that uncontrolled LPS-toll-like receptor 4 signalling response may result in excessive inflammation, which is a primary contributor to diffuse alveolar damage [5]. However, therapeutic strategies aimed at reducing the inflammatory response have shown limited clinical benefit to date [6-8]. Accordingly, it is necessary to further characterise the functions and mechanisms through which LPS causes alveolar epithelial cell damage.

Autophagy is a fundamental cell biological process that is responsible for the turnover of long-lived proteins and cytoplasmic organelles through a lysosome-mediated degradation pathway. During autophagy, the cytosol and organelles such as mitochondria and the endoplasmic reticulum (ER) are engulfed into a double- or multi-membrane-bound vacuole (an autophagosome). The vacuole membrane subsequently fuses with a lysosome, in which the encapsulated components are degraded by lysosomal hydrolases, and the breakdown products are released for reutilization [9-11]. This self-digestion process has a dual effect on cell survival: cytoprotection and cytotoxicity. Evidence has been presented that autophagy can promote cell survival or cell death depending on the cell type, the specific circumstances and stimuli present, and the duration and strength of the stimulus exposure [12, 13]. For instance, the autophagic protein microtubule-associated protein 1 light chain 3 (LC3B) can facilitate bronchial epithelial cell survival in the context of oxygen-dependent cytotoxicity [12], whereas avian influenza A (H5N1) infection induces autophagic cell death through the dysregulation of the Akt-TSC2-mTOR signalling pathway in alveolar epithelial A549 cells [14]. Notably, hypoxia-induced autophagy exerts distinct effects on the survival of different cell types. Furthermore, for the same cell types, hypoxia-induced autophagy can promote survival if induced under conditions of well-controlled hypoxia and without metabolic stress but can induce cell death under conditions of severe hypoxia concomitant with nutrient limitation and low pH [15, 16]. Previous studies have confirmed that exposure to LPS can decrease the viability of A549 cells [17, 18]. Caspase-dependent apoptosis has been well investigated as a pathway of cell death in these studies. However, it is not well-known whether autophagy plays a role in either the initiation or inhibition of cell death processes when human alveolar epithelial cells are challenged with LPS.

Many sources of stress cause a perturbation in the ER, termed ER stress, which results in the accumulation of unfolded or misfolded proteins. This accumulation subsequently triggers the unfolded protein response (UPR), which enables the cell to either overcome the stress or initiate cell death. Three UPR signal transduction pathways governed by three specific ER stress sensors, inositol requiring protein 1 (IRE1), activating transcription factor (ATF) 6, and double-stranded RNA-activated protein kinase-like ER kinase (PERK), have been identified [19, 20]. Under non-stress conditions, these sensors bind to the ER-resident glucose-regulated protein (GRP) chaperones (GRP78 and GRP94 are prominent ER chaperones) and exist in their deactivated forms [21-23]. Upon stress, misfolded proteins in the ER lumina cause the UPR sensors to detach from the chaperones, then freeing sensors activate downstream signalling pathways [24]. Activation of PERK results in the phosphorylation of eIF-2α, which reduces the synthesis of most cellular proteins but promotes the initiation of ATF4 translation, leading to the transcription of ATF4’s downstream targets, including growth arrest DNA damage gene 34 (GADD34). XBP-1 splicing and the cleavage of nuclear ATF6 represent the activities of the IRE1 and ATF6 pathways, respectively [25]. Evidence has also been presented indicating that there is important crosstalk between the ER stress and autophagy pathways because autophagy can be used as a misfolded protein degradation system [26]. The ER stressors tunicamycin and thapsigargin activate the formation of
autophagosomes in SK-N-SH neuroblastoma cells [27]. ER stress induced by tunicamycin, DTT or MG132 enhances autophagy via negative regulation of the AKT/TSC/mTOR pathway in MEF cells [28], and hypoxia enhances autophagy in MEF cells [29]. Specifically, the UPR triggered by the ER stress response to insults plays an important role in the activation of autophagy as well as in the determination of life-or-death cell fates [30]. However, whether LPS-induced autophagic activity is associated with ER stress and the UPR in A549 cells has not been established.

In the present study, using a cell-culture model of ALI [31-33], we provide novel evidence that LPS-induced autophagy promotes cell death in alveolar epithelial cells and that this process depends upon the activation of the PERK branch of the UPR upon ER stress.

Materials and Methods

Cell culture and drug treatment

The human type II lung epithelial A549 cell line was obtained from the American Type Culture Collection. Cells were maintained in Dulbecco’s modified Eagle’s medium F-12 supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and incubated at 37°C in a humidified incubator containing 5% CO₂.

LPS (0111:B4), 3-methyladenine (3-MA), rapamycin (Rapa), Z-VAD-FMK and 4-phenylbutyrate (4-PBA) were purchased from Sigma. Z-VAD-FMK (50 μM) or Rapa (100 nM) diluted with DMSO or 3-MA (3 mM) or 4-PBA (2.5 mM) dissolved in PBS were used to pretreat the cells for 2 h before the administration of LPS dissolved in PBS. LPS was pre-incubated at 37°C for 1 h with polymyxin B (100 g/ml) or PBS. The control cells received PBS or DMSO only.

Transfections and RNA interference

Cells were transiently transfected with an enhanced green fluorescent protein (EGFP)-LC3 plasmid (constructed by GeneChem Co. Ltd, Shanghai, China) using Lipofectamine 3000 Transfection Reagent (Invitrogen) according to the manufacturer’s instructions. After a 48-h transfection, the cells were treated with LPS at 50 μg/ml for another 16 h and then subjected to laser confocal scanning microscopy (TCSSP5; Leica Microsystems, Mannheim, Germany).

For the RNA interference experiments, A549 cells were transfected with Beclin-1 short interfering RNA (siRNA), Atg5 siRNA (Cell Signaling), PERK siRNA, ATF4 siRNA (Santa Cruz), or control siRNA or co-transfected with EGFP-LC3 plasmid using Lipofectamine 3000 Transfection Reagent (Invitrogen) as directed by the manufacturer. After 48 h, the effect of the siRNA treatment was assessed via Western blotting analysis. In parallel, cells were treated with LPS and analysed as described above.

Transmission electron microscopy

Cells were treated with LPS (50 μg/ml) or with an equal volume of control solution for 16 h, fixed in 2.5% glutaraldehyde at 4°C overnight and then postfixed with 2% osmium tetroxide for 1.5 h at room temperature. The cells were then embedded, and ultra-thin sections were obtained. After staining with uranyl acetate/lead citrate, the sections were analysed by transmission electron microscopy (JEM-1400PLUS, Japan) at 60 kV.

Cell death assay

A549 cells were seeded on 96-well plates at 5,000 and allowed to attach overnight. After the indicated drug treatment, cell viability was measured using the Cell Counting Kit-8 (CCK8) assay kit (Dojindo, Japan) according to the manufacturer’s instructions. Briefly, water-soluble tetrazolium salt (WST) 8 was added to each well, and the plates were incubated for 2 h. Absorbance values at 450 nm were then recorded with a microplate reader. The proportion of cells per treatment group was normalized to the control wells.

Apoptosis assay

Apoptotic cells were identified using the Annexin V/propidium iodide (PI) staining kit (BD Biosciences) according to the manufacturer’s instructions as previously described [18] and analysed with a FACSCalibur
flow cytometer (Becton Dickinson, San Jose, CA, USA). Both early apoptotic (Annexin V-positive, PI-negative) and late apoptotic (Annexin V-positive and PI-positive) cells were included in cell death determinations.

**Lactate dehydrogenase (LDH) assay**

After treatment with LPS (50 μg/ml) or with an equal volume of control solution for 16 h, the rate of LDH leakage in the A549 cells was measured using a kit (Beyotime) according to the manufacturer’s protocol.

**Immunoblotting**

Treated cells were washed with PBS and lysed in 1×NuPAGE LDS sample buffer (Invitrogen) to obtain total protein lysates. Nuclear fractionations were carried out using the Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) according to the manufacturer’s instructions. The protein concentration in the resulting fractions was measured using an enhanced BCA protein assay kit (Beyotime). The proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 and incubated with primary antibodies directed against the following proteins: LC3B, Atg5, Beclin1 and GADD34 from Sigma; p62, XBP-1 s, pPERK, p-eIF2α and ATF4 from Cell Signaling; ATF6 from Imgenex; and GRP78, GRP94, histone H1 and GAPDH from Santa Cruz. Horseradish peroxidase-conjugated, species-specific secondary antibodies (Kirkegaard and Perry Laboratories) were used to detect the primary antibodies. Bands were visualized with an enhanced chemiluminescence reagent (Perkin-Elmer Life Sciences).

**Statistical methods**

All values were expressed as the mean±SEM of the indicated number of experiments. The statistical significance of comparisons between 2 groups was evaluated using a 2-tailed Student’s t-test. P values of less than 0.05 were considered statistically significant.

**Results**

**LPS induced autophagy in A549 cells**

To evaluate autophagy in LPS-treated A549 cells, proteins involved in the induction or progression of the autophagic pathway, including LC3B, Beclin-1 and Atg5, were detected by immunoblotting. Treating cells with LPS resulted in an increase in total LC3B expression and in the dose- and time-dependent accumulation of its active form, LC3B-II. Consistent with the observed increase in LC3B expression, Beclin-1 and Atg5 were upregulated after LPS treatment. These events were also accompanied by the degradation of p62, an autophagic substrate (Fig. 1A). To verify that the increased autophagy was due to the presence of LPS, LPS was pre-incubated with polymyxin B, which binds to LPS and inhibits its activity. We found that pre-incubation of LPS with polymyxin B decreased the formation of LC3B-II protein (Fig. 1B).

Next, we examined the occurrence of EGFP-LC3-labeled structures (EGFP-LC3 puncta), which is a marker of autophagosome formation. LPS treatment induced the formation of EGFP-LC3 puncta in EGFP-LC3-transfected A549 cells (Fig. 1C, upper panel). The quantification of these results revealed that the number of LC3 puncta per cross-sectioned cell increased after the LPS treatment relative to the control treatment (Fig. 1C, lower panel).

To further confirm the presence of autophagy after treatment with LPS, we sought to identify morphological indices of autophagy via transmission electron microscopy. The accumulation of double-membrane autophagosomes was identified in the A549 cells treated with LPS, while this ultrastructural feature was rarely observed in the control cells (Fig. 1D). Taken together, these findings suggest that exposure to LPS triggers autophagy in A549 cells.

**LPS at 50 μg/ml inhibited cell viability independent of apoptosis and necrosis in A549 cells**

The effect of LPS on the viability of A549 cells was determined using a CCK-8 assay. Treatment of the cells with LPS at concentrations of 50 (Fig. 2A), 100, and 150 μg/ml (data
Fig. 1. LPS-induced autophagy in A549 cells. (A) A549 cells were treated without or with various concentrations of LPS for 16 h or treated with 50 μg/ml LPS for different lengths of time. Total cellular extracts were then prepared and subjected to immunoblotting analysis using antibodies against LC3B, Beclin-1, Atg5, p62 and GAPDH (as a loading control). The bar graph indicates the relative abundance of the proteins (normalized to that of GAPDH). *P < 0.05, **P < 0.01 in comparison to control group. (B) A549 cells were treated with LPS which was pre-incubated without or with polymyxin B. The level of the autophagic protein LC3B were determined by immunoblotting. The bar graph indicates the relative abundance of the LC3-II protein (normalized to that of GAPDH). **P<0.01. (C) A549 cells transfected with the EGFP-LC3 plasmid were treated without or with LPS (50 μg/ml) for 16 h. Upper panel: LC3 punctae visualized by confocal laser scanning microscopy. Lower panel: Quantification of the number of LC3 puncta per cross-sectioned cell. The data are presented as the mean±SEM of 3 independent experiments. **P<0.01. (D) Transmission electron microscopy images of A549 cells treated without or with LPS (50 μg/ml) for 16 h. Bar =1 μm.

not shown) resulted in significant decreases in cell viability at 16 h, whereas the treatment of the cells with LPS at 0.1, 1, 10, and 25 μg/ml did not affect cell viability (Fig. 2A). A time-course analysis indicated that 50 μg/ml LPS decreased cell viability at 16 h post-treatment
but that viability at 2, 4, and 8 h was not affected (Fig. 2B). Therefore, 50 µg/ml seemed to be the minimal cytotoxic dose of LPS for A549 cells. The effect of LPS on cell death is specific because pretreatment of LPS with polymyxin B increased the viability of A549 cells (Fig. 2C). This result is consistent with the results reported by Peter S. Tang et al. [17].

In addition, the effect of 50 µg/ml LPS on the apoptosis of A549 cells was analysed via double-labelled flow cytometry using annexin-V/PI. Camptothecin (4 µM) was used as a positive control for apoptosis [34]. We found that treatment with 50 µg/ml LPS for 16 h did not significantly induce the apoptosis of A549 cells (Fig. 2D), whereas this dose of LPS increased apoptosis at 48 h after the stimulation (data not shown). Furthermore, 50 µg/ml LPS did not affect the rate of lactate dehydrogenase (LDH) leakage, a hallmark of cell necrosis, at 16 h compared to the control (Fig. 2E). Taken together, these findings suggest that the LPS-induced decrease in cell viability is not attributable to apoptosis or necrosis. Therefore, we presume that other mechanism(s) may contribute to the observed cell death.
Fig. 3. Autophagy is required for LPS-induced A549 cell death. (A) A549 cells pretreated with 3-MA (3 mM) or Rapa (100 nM) for 2 h followed by treatment with LPS (50 μg/ml) for 16 h. Total protein lysates were analysed by immunoblotting with antibodies against LC3B and GAPDH (as a loading control). The bar graph indicates the relative abundance of the LC3-II protein (normalized to that of GAPDH) in three experiments. (B) A549 cells were pretreated with the autophagy inhibitor 3-MA (3 mM), the autophagy inducer Rapa (100 nM) or the apoptosis inhibitor Z-VAD (50 μM) for 2 h followed by treatment with LPS (50 μg/ml) for 16 h. Cell viability was determined by a CCK8 assay. (C and D) The effectiveness of the knockdown of Beclin-1 or Atg5 and the autophagic protein LC3 were determined by immunoblotting. The bar graph indicates the relative abundance of the LC3-II protein (normalized to that of GAPDH). (E) A549 cells were co-transfected with EGFP-LC3 and a control siRNA or an siRNA specific for Beclin-1 or Atg5 for 48 h followed by treatment with LPS (50 μg/ml) for 16 h. Upper panel: LC3 vesicle fluorescence was visualized with a laser confocal scanning microscope. Lower panel: Quantification of the number of LC3 puncta per cross-sectional cell. (F) A549 cells were transfected with a control siRNA or with an siRNA specific for Beclin-1 or Atg5 for 48 h followed by treatment with LPS (50 μg/ml) for 16 h; cell viability was then determined by CCK8 assay. The data are presented as the mean±SEM of 3 independent experiments. *P<0.05; **P<0.01.

Autophagy is required for LPS-induced A549 cell death

We used a specific pharmacological inhibitor of autophagy, 3-MA, and the autophagy inducer rapamycin to examine the effects of autophagic regulators on the LPS-induced death of A549 cells. We observed that the increase in the level of LC3B-II protein was reversed by pretreatment with 3-MA, whereas the formation of LC3B-II was significantly upregulated when LPS-stimulated A549 cells were pretreated with Rapa (Fig. 3A). These findings suggest that LPS-induced autophagy in A549 cells can be regulated by both 3-MA and Rapa. Furthermore, the decrease in the viability of A549 cells exposed to 50 μg/ml LPS for 16 h was efficiently abrogated by pretreatment with 3-MA. In contrast, Rapa led to a further decrease in the viability of A549 cells treated with LPS. Additionally, pretreatment with the
apoptosis inhibitor Z-VAD failed to prevent cell death in A549 cells that had been treated with LPS at 50 µg/ml for 16 h ($P=0.17792$) (Fig. 3B).

To further confirm the functional role of autophagy in LPS-induced cell death in human alveolar epithelial cells, the expression of Beclin-1 or Atg5, two critical genes involved in the autophagy pathways, was interrupted in A549 cells. We observed the expected silencing of the target gene expression in A549 cells treated with siRNAs specific for Beclin-1 or Atg5 (Fig. 3C). Furthermore, as shown in Fig. 3D and 3E, the knockdown of Beclin-1 or Atg5 decreased the expression level of LC3-II protein and the number of LC3 puncta in the A549 cells treated with LPS. Moreover, the decrease in cell viability after exposure to LPS was efficiently abrogated in A549 cells undergoing Beclin-1 or Atg5 knockdown (Fig. 3F).

**ER stress contributes to LPS-induced autophagy in A549 cells**

To evaluate whether ER stress is responsible for the activation of autophagy in A549 cells in response to LPS, we monitored the expression of two ER stress markers, the GRP78 and GRP94 proteins, in LPS-stimulated A549 cells. As illustrated in Fig. 4A, the protein expression levels of both GRP78 and GRP94 were upregulated after LPS treatment in a dose- and time-dependent manner. In addition, in A549 cells exposed to 50 µg/ml LPS for 16 h, the upregulation of GRP78 and GRP94 was reduced by pretreating the cells with the ER
stress inhibitor 4-PBA, which was accompanied by a decrease in the level of LC3B-II (Fig. 4B). The results indicate that ER stress contributed to the activation of autophagy in LPS-treated A549 cells.

**LPS activated the PERK-\(\text{eIF2}\alpha-\text{ATF4}-\text{GADD34} \) pathway of the UPR but had no effect on the ATF6 and IRE1-\(\text{sXBP1} \) pathways.**

To assess whether all three branches of the UPR are activated upon LPS-induced stress in A549 cells, we analysed constituents involved in these three pathways of UPR signalling.
Fig. 6. The PERK-ElF2α-ATF4-GADD34 pathway is responsible for LPS-induced autophagy and cell death in A549 cells. A549 cells were transfected with a control siRNA or an siRNA specific for PERK or ATF4 for 48 h followed by treatment with LPS (50 μg/ml) for 16 h. The effectiveness of the PERK or ATF4 knockdown (A and C) and the level of the autophagic protein LC3B were determined by immunoblotting. The bar graph indicates the relative abundance of the LC3-II protein (normalized to that of GAPDH) (B and D). A549 cells were co-transfected with EGFP-LC3 and with a control siRNA or an siRNA specific for PERK or ATF4 for 48 h followed by treatment with LPS (50 μg/ml) for 16 h. The fluorescence of the LC3 puncta was visualized with a laser confocal scanning microscope (E). The quantification of the number of LC3 puncta per cross-sectioned cell is shown (F). Cell viability was determined by CCK8 assay (G). The data are presented as the mean±SEM of three independent experiments. *P<0.05; **P<0.01.

We found that LPS treatment resulted in the accumulation of phosphorylated PERK (p-PERK) and constituents of the PERK cascade, phosphorylated ElF2α (p-ElF2α), nuclear ATF4 and GADD34. However, there were hardly any changes in the splicing of XBP1 or in the nuclear cleaved ATF6 in LPS-treated A549 cells compared with the control cells (Fig. 5A and 5B).
The PERK-eIF2α-ATF4-GADD34 pathway is responsible for LPS-induced autophagy and cell death in A549 cells

We next investigated whether the suppression of the PERK pathway would affect the LPS-induced autophagy and cell survival in A549 cells. The transfection of the cells with an siRNA targeted against PERK or ATF4 inhibited the expression of the proteins (Fig. 6A and 6C). In addition, the knockdown of PERK or ATF4 decreased the formation of LC3B-II (Fig. 6B and 6D) and the punctate accumulation of LC3 (Fig. 6E and 6F), as well as partially increased the viability of LPS-treated A549 cells (Fig. 6G).

Discussion

Alveolar epithelial cell death is a prominent characteristic of the ALI induced by LPS and has been demonstrated to play a critical role in the development of ALI [35, 36]. In the current study, employing the cell culture model of ALI used by many research groups, which utilises A549 cells to examine the injury-defence mechanism of alveolar epithelial cells [31-33], we elucidated the effects of autophagy on the outcome of human alveolar epithelial cell fate in response to LPS and studied the underlying mechanisms.

Autophagy, an evolutionarily conserved process, has primarily been characterized as a cellular homeostatic mechanism that operates during states of nutrient deficiency [37, 38]. In addition, many types of stressors, including infectious pathogens and their components, have recently been confirmed as autophagy inducers in professional or non-professional phagocytic cells [39-41, 14]. Consistent with these findings, our present results indicate that LPS can induce autophagy in A549 cells, as evidenced by the observation of both biochemical and morphological indices of autophagy in the LPS-treated A549 cells, including the enhanced expression of autophagic proteins, the decreased abundance of an autophagic substrate accompanied by the formation of double-membrane autophagosomes that were detected by electron microscopy, and the accumulation of EGFP-LC3 puncta in transfected cells observed with a laser confocal scanning microscope. The effect of LPS on autophagy in A549 cells is specific because blocking LPS with polymyxin B rescued the formation of the autophagic protein LC3B-II.

Autophagy also has been considered to form part of a pro-death or a pro-survival pathway in a variety of models [42]. In some cases, autophagy represents an adaptive response that protects a cell from death induced by exogenous or endogenous stimuli [12, 43-46]. In contrast, in other cellular environments, autophagy can act as an alternative mechanism to promote a type of cell death that is termed autophagic cell death (also known as type II programmed cell death) [14, 39, 47, 48]. It appears that autophagic cell death, which is often observed under extreme stress conditions, could be considered the outcome of failed adaptation [15]. Based upon our observations and these concepts, we hypothesized that autophagy may determine cell fate when human alveolar epithelial A549 cells are exposed to LPS. We therefore investigated the possible functional roles of autophagy in LPS-induced A549 cell death. In our model, LPS treatment induced autophagic cell death in the A549 cells, based on the following findings: (I) a minimal cytotoxic dose of LPS inhibited cell survival but did not induce apoptosis or necrosis. The effect of LPS on cell death was specific because it was blocked by an LPS inhibitor; (II) the LPS-induced cell death was suppressed by genetic or pharmacologic inhibition of autophagy; (III) the LPS-induced cell death was enhanced by autophagy inducer; and (IV) cell viability was not efficiently rescued simply by treatment with an inhibitor of apoptosis. From these findings, we infer that increased autophagy is an alternative or complementary mechanism by which LPS results in alveolar epithelial cell injury and contributes to the pathogenesis of ALI.

It should be noted that LPS was previously reported to induce apoptosis in A549 cells [17, 18, 31, 49], which differs from the current results. We suspect that the discrepancy can be attributed to the varied duration and strength of the LPS exposure in the different experimental models used in these studies. Cells undergoing different degrees of stimulation...
induced by LPS may undergo different death patterns. Another possible explanation is that A549 cells were resistant to the induction of apoptosis in the absence of co-culturing the cells with polymorphonuclear leukocytes [31].

To date, the exact mechanism regulating the autophagy induced by LPS has not been fully clarified. Recently, accumulating data have provided evidence that ER stress serves as an important trigger of autophagy. Under most conditions, the UPR's three known sensors are coordinately activated [24]. In this study, we found that LPS initiated ER stress in A549 cells, as indicated by the upregulation of hallmarks of ER stress, the GRP78 and GRP94 proteins. Similar findings were recently reported in LPS-induced normal human bronchial epithelial (NHBE) cells [50]. Furthermore, in our model, the ER stress inhibitor 4-PBA efficiently reversed the LPS-induced formation of LC3-II in A549 cells. These findings suggest that ER stress contributes to the activation of autophagy in LPS-treated A549 cells. We next sought to determine whether all three branches of the UPR were elicited following LPS-induced stress in A549 cells. Unexpectedly, we found that LPS treatment of A549 cells activated one transducer of UPR, PERK, and its downstream products p-eIF2α, ATF4 and GADD34; however, LPS but rarely alter the other two branches of the UPR as reflected by XBP-1 splicing and cleaved nuclear ATF6. These results demonstrate that LPS selectively activates the PERK-eIF2α-ATF4-GADD34 pathway of the UPR during ER stress in A549 cells.

According to recent evidence, various ER stressors result in the activation of distinct branches of the UPR accompanied by differential cellular effects in different cell types [30, 51, 52]. In addition, the impact of ER stress on cell fate depends on the level of activation of the UPR. For instance, mild ER stress that selectively elicits activation of the XBP1 branch of the pathway has been shown to play a neuroprotective role in a model of Parkinson's disease [53], whereas severe ER stress followed by a sustained and prolonged UPR that involves CHOP activation often leads to cell death [54]. Therefore, we further assessed whether the increased autophagy and decreased survival of LPS-treated A549 cells were associated with the preferential activation of the PERK pathway. As expected, we found that inhibiting the PERK pathway via the genetic interruption of PERK or ATF4 attenuated the formation of LC3-II and decreased the punctate accumulation of LC3, accompanied by a partial enhancement of the viability in the A549 cells exposed to a minimal cytotoxic dose of LPS. Our data indicate that the PERK pathway regulates autophagy activation during ER stress and that it promotes cell death when the A549 cells are challenged with a minimal cytotoxic dose of LPS.

In conclusion, our present findings demonstrate that alveolar epithelial cells can undergo autophagic cell death following a challenge with a minimal cytotoxic dose of LPS and that this process depends upon the activation of the PERK pathway of the UPR upon ER stress. These molecular events might represent another important mechanism contributing to the alveolar epithelial cell damage induced by LPS, which likely results in the pathogenesis of ALI. These findings may provide potential clues for exploiting possible therapeutic drugs for the management of alveolar epithelial cell death during ALI.

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**Disclosure Statement**

The authors declare no conflicts of interest.
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