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Involvement of the MAPK and PI3K pathways in chitinase 3-like 1-regulated hyperoxia-induced airway epithelial cell death

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ABSTRACT

Background: Exposure to 100% oxygen causes hyperoxic acute lung injury characterized by cell death and injury of alveolar epithelial cells. Recently, the role of chitinase 3-like 1 (CHI3L1), a member of the glycosyl hydrolase 18 family that lacks chitinase activity, in oxidative stress was demonstrated in murine models. High levels of serum CHI3L1 have been associated with various diseases of the lung, such as asthma, chronic obstructive pulmonary disease, and cancer. However, the role of CHI3L1 in human airway epithelial cells undergoing oxidative stress remains unknown. In addition, the signaling pathways associated with CHI3L1 in this process are poorly understood.
Purpose: In this study, we demonstrate the role of CHI3L1, along with the MAPK and PI3K signaling pathways, in hyperoxia-exposed airway epithelial cells.
Method: The human airway epithelial cell line, BEAS-2B, was exposed to >95% oxygen (hyperoxia) for up to 72 h. Hyperoxia-induced cell death was determined by assessing cell viability, Annexin-V FITC staining, caspase-3 and -7 expression, and electron microscopy. CHI3L1 knockdown and overexpression studies were conducted in BEAS-2B cells to examine the role of CHI3L1 in hyperoxia-induced apoptosis. Activation of the MAPK and PI3K pathways was also investigated to determine the role of these signaling cascades in this process.
Results: Hyperoxia exposure increased CHI3L1 expression and apoptosis in a time-dependent manner. CHI3L1 knockdown protected cells from hyperoxia-induced apoptosis. In contrast, CHI3L1 overexpression promoted cell death after hyperoxia exposure. Finally, phosphorylation of ERK1/2, p38, and Akt were affected by CHI3L1 knockdown.
Conclusion: This study indicates that CHI3L1 is involved in hyperoxia-induced cell death, suggesting that CHI3L1 may be one of several cell death regulators influencing the MAPK and PI3K pathways during oxidative stress in human airway epithelial cells.

1. Introduction

Exposure to high oxygen levels is inevitable for preterm babies or patients suffering from respiratory failure. However, prolonged exposure to such high amounts of oxygen causes hyperoxic acute lung injury, which is characterized by alveolar endothelial and epithelial cell death [1,2]. Apoptosis has been described as a major death mechanism in hyperoxia-induced lung injury *in vivo* and *in vitro* [3,4]. Controlled by intercellular signaling, apoptosis also plays a critical role in tissue remodeling and homeostasis [5,6]. Caspases, which constitute a family of cysteine proteases, are the central regulators of apoptosis. Numerous studies have established that caspases -8, -9, and –10 play important roles in initiating apoptosis by coupling cell death stimuli to the downstream effector caspases, namely caspase-3, -6, and -7. Once activated, these effector caspases execute apoptosis by cleaving cellular proteins at specific aspartate residues [7,8].

Chitinase 3-like 1 (CHI3L1; also known as YKL-40), a member of the evolutionarily conserved glycosyl hydrolase 18 family, is produced by a variety of mammalian cells, including chondrocytes, synovial cells, endothelial cells, and epithelial cells [9–11]. Increased CHI3L1 levels have been associated with various pathological conditions such as bacterial infections, rheumatoid arthritis,

Abbreviations: CHI3L1, chitinase 3-like 1; MAPK, mitogen-activated protein kinase; PI3K, phosphotidylinositol-3-kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal protein kinase.

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allergic disease, and a variety of malignancies [12,13]. In the lungs, high serum CHI3L1 level has been linked to higher incidences of asthma, chronic obstructive pulmonary disease, and lung cancer [14–16]. Ober et al. reported that *CHI3L1* indicates an increased susceptibility to asthma, bronchial hyper-responsiveness, and reduced lung function. In fact, elevated levels of circulating CHI3L1 is used as a biomarker for asthma and reduced lung function [14]. Recently, CHI3L1 and its mouse homolog BRP-39 were demonstrated to be critical regulators of oxidant injury, inflammation, and epithelial apoptosis in murine and human lungs [17]. The novel regulatory role of BRP-39/CHL13L1 in cigarette smoke-induced inflammation and emphysematous destruction was also reported [18]. These studies indicate the pivotal role of CHI3L1 in airway inflammation and oxidative stressed lung. Despite this and the established importance of human airway epithelial cells as an essential alveolar capillary barrier and the first line of defense in immunity, the role of CHI3L1 in human airway epithelial cells undergoing oxidative stress has yet to be fully elucidated. Moreover, the signaling pathways involved in CHI3L1-regulated hyperoxia are poorly understood. Therefore, in this study, we demonstrate the role of CHI3L1, along with the MAPK and PI3K signaling pathways, in hyperoxia-exposed airway epithelial cells.

2. Materials and methods

2.1. Cell culture and hyperoxia exposure

The human airway epithelial cell line BEAS-2B was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in bronchial epithelial growth medium (BEGE; Walkersville, MD). Cells were grown under humidified conditions consisting of 95% air and 5% CO_2 at 37 °C. For hyperoxia exposure, cells were plated in an MIC-101 chamber (Modular Incubator, Billups-Rothenberg Inc., Germany) filled with 95% O_2 and 5% CO_2 for up to 72 h at 37 °C. The concentration of O_2 in the chamber was monitored by MaxO_2 (Maxtec, Inc.). As a control group, normoxia cells were incubated without the chamber under 95% air and 5% CO_2 at 37 °C. The gases were replaced every day.

2.2. Cell viability determination

Cell viability was measured using the Cell Count Kit-8 (Sigma–Aldrich, St. Louis, MO). Cells were seeded at 1×10^3 cells per well in 96-well culture plates and incubated overnight. Cells were either left untreated or exposed to hyperoxia for up to 72 h. After the indicated time, a CCK-8 solution (WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodiumsalt) was added and the plates were incubated for another 2 h before absorbance were measured at 450 nm.

2.3. Annexin V/propidium iodide staining

Apoptosis was determined using the Annexin V-FITC kit (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. Flow cytometry was conducted using a FACS LSR II (BD Biosciences) and flow cytometric analysis was performed using FlowJo software (TreeStar, Ashland, OR).

2.4. Electron microscopy

Cells were fixed in phosphate buffer containing 2% glutaraldehyde and 2% paraformaldehyde after treatment with either hyperoxia or normoxia. The cells were photographed using a JEOL JEM-1011 transmission electron microscope (JEOL, Peabody, MA).

2.5. Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA) prior to complementary DNA synthesis. PCR amplification was carried out using specific primer pairs. GAPDH was used as an internal standard. The primer sequences used were obtained from Primer Bank (http://pga.mgh.harvard.edu/primerbank) and were as follows: CHI3L1: 5′-CCA AGG AGC CAA ACA TCC TA-3′ (sense) and 5′-GAA GGG GAA GTA GGA TAG GGG-3′ (antisense); caspase 3: 5′-ATG GAA GCG AAT CAA TGG ACT C-3′ (sense) and 5′-CTG TAC CAG ACC GAG ATG TCA-3′ (antisense); caspase 7: 5′-CGG TCC TCG TTT GTA CCG TC-3′ (sense) and 5′-GGT GGT CTT GAT GGA TCG CA-3′ (antisense); GAPDH: 5′-CCC CTT CAT TGA CCT CAA CT-3′ (sense) and 5′-GAT GAC AAG CTT CCC GTT CT-3′ (antisense). Fold changes were calculated using the comparative ΔΔCt method.

2.6. Western blot analysis

Cell lysates were subjected to Western blot analysis with specific antibodies against CHI3L1 (Santa Cruz Biotechnology, Santa Cruz, CA), cleaved caspase-3, cleaved caspase-7, ERK1/2, p-ERK1/2, JNK, p-JNK, p38, p-p38, Akt, p-Akt, and β-actin (Cell Signaling Technology, Beverly, MA). Total protein was extracted using a Mammalian Protein Extraction reagent (Pierce, Rockford, IL). Then, the lysates were subjected to sodium dodecyl sulfate polyacrylamide gels, and electrotransferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking with 5% skimmed milk in 1× Tris buffered saline containing 0.1% Tween 20 for 1 h, the membranes were incubated overnight at 4 °C with primary antibody followed by horseradish peroxidase-conjugated anti-goat (Santa Cruz Biotechnology) or anti-rabbit secondary antibody (Cell Signaling Technology). Protein bands were visualized using an enhanced chemiluminescence kit (Amersham International, UK) and exposed to hyperfilm (Amersham) or the ImageQuant™ LAS 4000 Mini Biomolecular Imager (GE Healthcare, Sweden).

2.7. Cell transfection

The CHI3L1-expressing vector or an empty pcDNA3 vector were transfected into BEAS-2B cells using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. Transiently transfected cells were incubated for an additional 24 h and exposed to either hyperoxia or normoxia.

2.8. Lentiviral short hairpin RNA (shRNA) production and transduction

CHI3L1 knockdown was accomplished by shRNA lentiviral transduction. CHI3L1 lentiviral glycerol stock was purchased from Sigma–Aldrich (GenBank ID: NM_001276). Lentiviruses were prepared by transient transfection of 293T cells using a liposomal cotransfection method. Transduction into BEAS-2B cells was performed in bronchial epithelial cell basal medium supplemented with 8 μg/mL polybrene (hexadimethrine-bromide, Sigma–Aldrich) according to the manufacturer's protocol. Before transduction, a puromycin titration was performed to select for lentivirus-encoding cells.

2.9. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments. Statistical analysis comparing the treated and control groups was assessed using the Student t test, where $p < 0.05$ was considered statistically significant.

3. Results

3.1. Hyperoxia induces apoptosis in human airway epithelial cells

To investigate hyperoxia-induced cell death, BEAS-2B cells were exposed to hyperoxia (95% O_2 and 5% CO_2) for 24, 48, and 72 h and cell viability was measured. As shown in Fig. 1A, cell viability declined rapidly following hyperoxia exposure in a time-dependant manner. To evaluate whether hyperoxia induces apoptosis or necrosis in airway epithelial cells, hyperoxia exposed cells were analyzed by flow cytometry after staining with Annexin-V FITC. As shown in Fig. 1B, the rate of apoptotic cells increased gradually for the first 48 h before increasing dramatically up to 72 h post-exposure. To confirm the induction of apoptosis by hyperoxia, we determined the levels of caspase expression and activation by real-time PCR and Western blot analysis, respectively (Fig 1C and D). Our data demonstrate that the levels of mRNA and activated caspases-3 and -7 proteins were upregulated in a time-dependent manner, with the greatest increase observed at 72 h. In addition, we confirmed apoptosis morphologically using electron microscopy. Hyperoxia-exposed cells exhibited morphological characteristics of apoptosis, including cell shrinkage, nuclear condensation, and membrane blebbing (Fig. 1E). These results suggest that hyper-oxygen stimulation induces apoptosis and that both caspase-3 and -7 are pivotal in hyperoxia-induced airway epithelial cell death.

3.2. Hyperoxia induces CHI3L1 expression

To demonstrate the involvement of CHI3L1 in hyperoxia-induced cell death, we first examined the expression of CHI3L1 after treatment with hyperoxia or normoxia for up to 72 h. Hyperoxia enhanced CHI3L1 production significantly. CHI3L1 mRNA transcription was increased by approximately 2.7- and 5.2-fold at 48 and 72 h, respectively, compared with the time-matched control (Fig. 2A). Similarly, the result of Western blot analysis showed

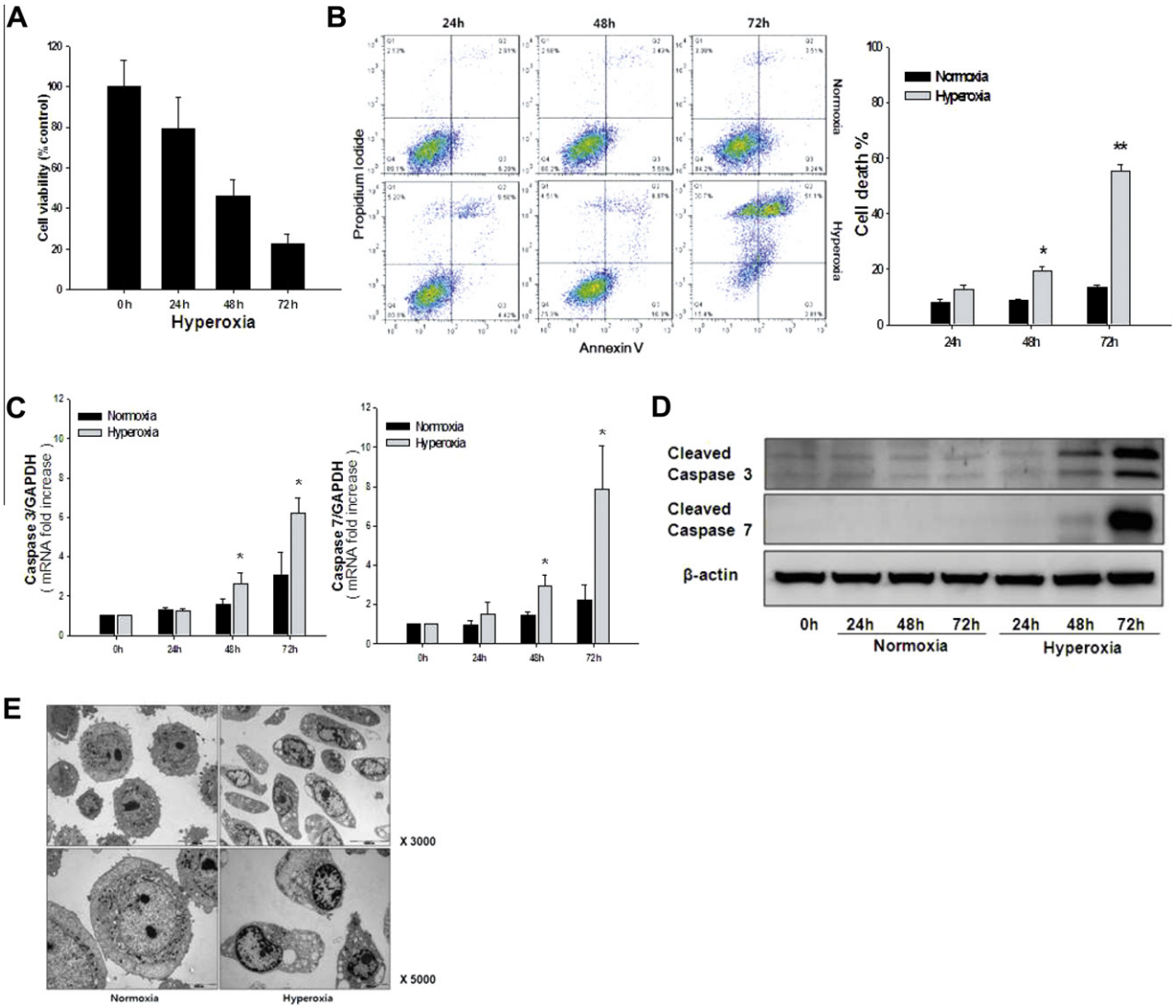

Fig. 1. Hyperoxia-induced apoptosis in BEAS-2B cells. BEAS-2B cells were exposed to hyperoxia (95% O_2 and 5% CO_2) or normoxia (95% air and 5% CO_2) for 24–72 h. (A) Cell viability was measured at the indicated times. The results are presented as a percentage of the time-matched control sample exposed to normoxia. (B) Cells were stained with Annexin V-FITC and analyzed by flow cytometry. Representative dot plots of flow cytometric analysis (Left). Quantitation of apoptotic cells corresponds to upper right and lower right in the graph (Right). The graph depicts the mean ± SEM of the three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ as determined by comparing control versus hyperoxia-exposed samples. (C) Caspase-3 and -7 mRNA were measured by real-time PCR. Data represent the mean ± SEM of the three independent experiments. $^{*}p < 0.05$ as determined by control versus hyperoxia-exposed samples. (D) Levels of cleaved caspase-3, cleaved caspase-7, and β-actin protein were assessed by Western blot analysis. The results shown are representative of three independent experiments. (E) Electron micrographs shows the ultra-structure of BEAS-2B cells treated with either hyperoxia (95% O_2 and 5% CO_2) or room air (95% air and 5% CO_2) for 55 h. Left: normoxia and right: hyperoxia exposed cells.

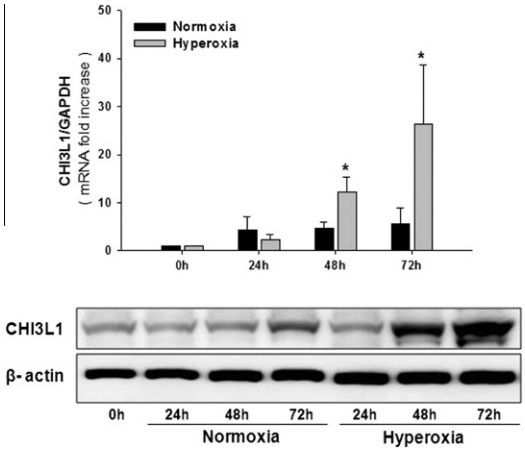

Fig. 2. Regulation of CHI3L1 expression by hyperoxia. BEAS-2B cells were exposed to hyperoxia or normoxia for 24–72 h. After exposure, cell lysates were prepared at the indicated times and the levels of CHI3L1 mRNA and protein was evaluated. (A) CHI3L1 mRNA was analyzed by real-time PCR. The data represent the mean ± SEM of four independent experiments. $^{*}p < 0.05$ as determined by control versus hyperoxia-exposed samples. (B) CHI3L1 protein was assessed by Western blot. The results shown are representative of three independent experiments.

time-dependent CHI3L1 accumulation (Fig. 2B). CHI3L1 protein was initially detected after 48 h and was clearly evident after 72 h of exposure to hyperoxia.

3.3. CHI3L1 augmented hyperoxia-induced cell death

To address the role of CHI3L1 in hyperoxia-induced cell death, lentiviral shRNA transduction was conducted. After transduction of shRNA-containing lentivirus that with either an anti-sense sequence of CHI3L1 or non-specific targeting sequence, BEAS-2B cells were exposed to hyperoxia for 24, 48, and 72 h. As shown in Fig. 3A and B, the CHI3L1 shRNA dramatically decreased CHI3L1 expression in these cells. To ascertain the impact of CHI3L1 knockdown on hyperoxia-induced cell death, caspase-3 and -7 expression were assessed by real-time PCR and Western blot (Fig. 3C and D). Our results demonstrate that CHI3L1 knockdown protected the airway epithelial cells against hyperoxia-induced caspase-3 and -7 activation. The level of activated caspase-3 and -7 was significantly lower in CHI3L1-silenced cells compared to that in control cells after 72 h of hyperoxia exposure.

Next, we examined the viability of the shRNA-containing cells (Fig 3E). Consistent with the reduced levels of caspase-3 and -7, cell viability was also increased in CHI3L1-silenced cells compared to control cells. These results suggest a role for CHI3L1 in regulating caspase activation after hyperoxia exposure in BEAS-2B cells. To further validate this function, CHI3L1 was overexpressed in BEAS-2B cells. As shown in Fig. 3F, CHI3L1-overexpressing cells exhibited a distinct increase in cleaved caspase-3 and -7 levels after hyperoxia exposure for 48 h compared with non-transfected and empty vector-transfected cells. Taken together, these results corroborate the regulatory function of CHI3L1 in augmenting airway epithelial cell death after hyperoxia exposure.

3.4. ERK1/2, p38, and Akt pathways are associated with CHI3L1 function

We next explored the signaling pathways through which CHI3L1 performs its regulatory role following hyperoxia exposure in BEAS-2B cells. The MAPK and PI3K-Akt pathways have been reported to play critical roles in hyperoxia-induced apoptosis. Thus, we examined the phosphorylation level of ERK1/2, JNK, p38, and Akt in BEAS-2B cells after hyperoxia exposure to determine the involvement of these pathways in our system (Fig. 4A). Hyperoxygen exposure enhanced the phosphorylation of ERK1/2 and p38 but reduced that of Akt. However, JNK phosphorylation was not detected.

To further confirm involvement of the MAPK and PI3K pathways in CHI3L1-regulated cell death in BEAS-2B cells, we investigated the effect of CHI3L1 knockdown on activation of the MAPK and PI3K pathways. As shown in Fig. 4B, ERK1/2 and Akt phosphorylation were increased significantly while p38 phosphorylation was abrogated in CHI3L1-deficient cells. These results suggest that ERK1/2, p38, and Akt participate in the function of CHI3L1 in hyperoxia-exposed BEAS-2B.

4. Discussion

In this study, we demonstrated the induction of apoptosis, as well as CHI3L1 expression, in BEAS-2B cells following hyperoxia exposure. Although apoptosis is known as a predominant feature of hyperoxia-induced cell death, hyperoxia-induced epithelial cell death has been shown to involve both apoptosis and necrosis. Lung epithelial cell apoptosis is a distinguishing characteristic of hyperoxia-induced acute lung injury [19]. *In vitro* studies have demonstrated that oxygen-exposed cells exhibit oxidation of DNA, lipids, and protein, as well as growth inhibition followed by cell death after prolonged exposure [20,21]. The biochemical and morphological features of apoptosis have been detected in hyperoxia-induced murine macrophages, fibroblastic cell lines, primary lung fibroblasts, and endothelial cells [22–24]. In contrast, hyperoxia-induced cell death in cultured human A549 and MLE12 cells exhibit the morphological features characteristic of necrosis, along with the involvement of both apoptosis- and necrosis-related signaling pathways [1,25]. Our present studies illustrate that BEAS-2B cells displayed the morphological and biochemical characteristics of apoptosis after hyperoxia exposure. In particular, hyperoxia-exposed BEAS-2B cells were smaller and exhibited nuclear condensation and membrane blebbing. Cleaved caspase-3 and -7 levels were also increased significantly by hyperoxia treatment, suggesting that hyperoxia-induced apoptosis is coordinated by members of the caspase family of cysteine proteases.

CHI3L1 is a chitinase-like protein that belongs to the evolutionarily conserved glycosyl hydrolase 18 family. Elevated levels of CHI3L1 in serum or tissue are strongly associated with various diseases, thereby making CHI3L1 a useful prognostic or diagnostic marker [26,27]. However, our understanding of the molecular processes regulating CHI3L1 induction and function remains incomplete. In this study, we demonstrate the expression of CHI3L1 and its regulatory role in hyperoxia-induced airway epithelial cell death. Our results showed that CHI3L1 expression in response to hyperoxia exposure is time-dependent in BEAS-2B cells. Furthermore, CHI3L1 knockdown protected airway epithelial cells against hyperoxia-induced caspase-3 and -7 activation. In contrast, CHI3L1 overexpression resulted in higher levels of cleaved caspase-3 and -7 compared with that of control cells. These results contradict a previous study that investigated CHI3L1 expression and function *in vivo*. Exposure to 100% O_2 led to a significant reduction in the expression and production of BRP-39, the mouse homolog of CHI3L1 [17]. In that study, BRP-39 null mice displayed exaggerated responses to hyperoxia, manifested as augmented alveolar-capillary permeability, tissue oxidation, neutrophil- and macrophage-rich inflammation, epithelial apoptosis, and premature cell death. Similarly, in a murine cigarette smoke exposure model in which oxidants play a major role in tissue injury, BRP-39/CHI3L1 accumulation was observed. In fact, the cigarette smoke-induced structural cell death response and emphysematous destruction were significantly enhanced in the absence of BRP-39 [18]. These

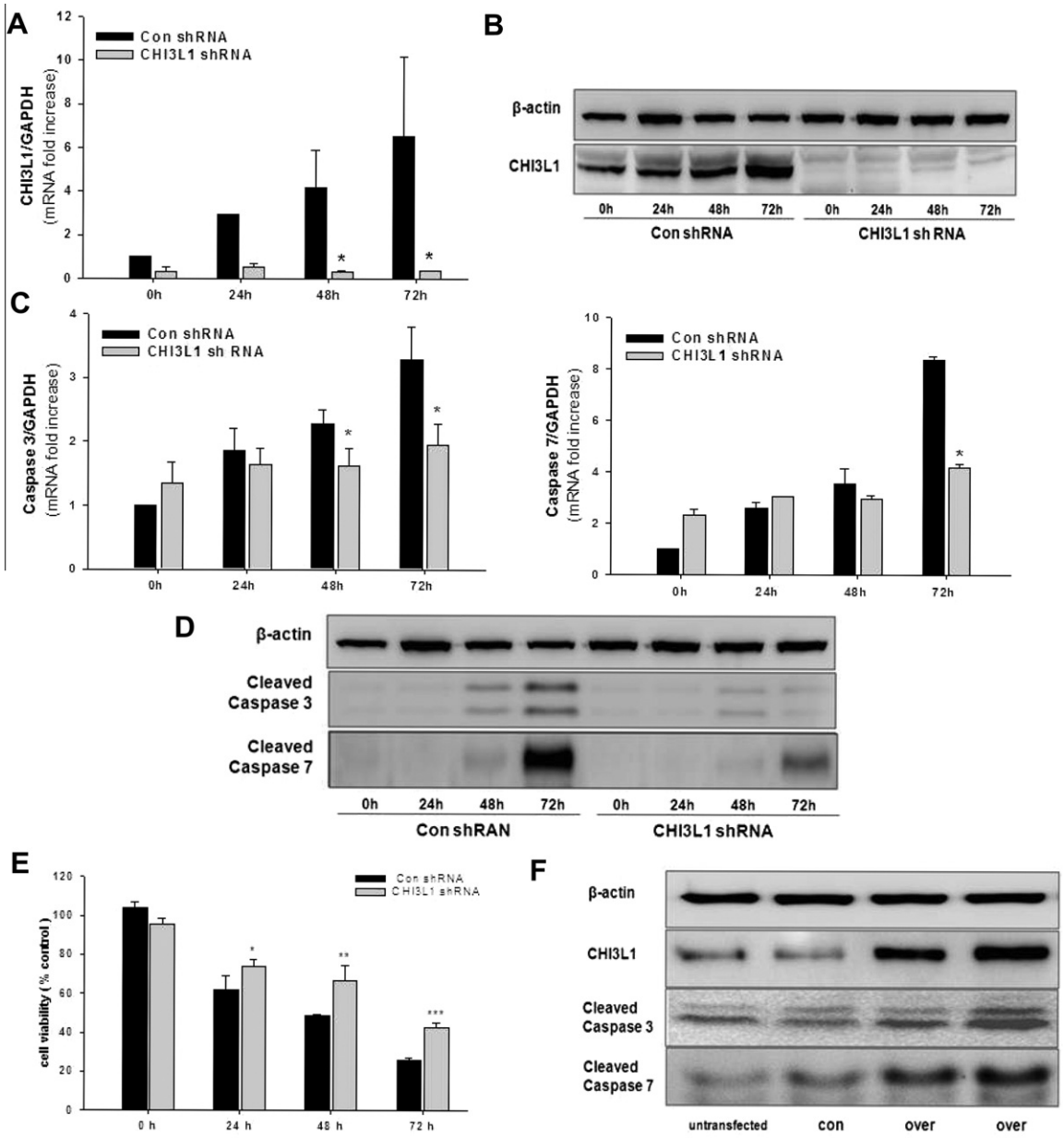

Fig. 3. Role of CHI3L1 in hyperoxia-induced caspase activation. Lentiviral shRNA transduction was conducted with shRNA targeted against an anti-sense sequence of CHI3L1 or a non-specific sequence and then exposed to hyperoxia for 24, 48, and 72 h. After exposure, cell lysates were prepared at the indicated times (A–E). (A and B) The levels of CHI3L1 mRNA and protein were assessed by real-time PCR and Western blot analysis, respectively. (C and D) Levels of caspase-3 and -7 mRNA and protein were measured by real-time PCR and Western blot analysis, respectively. (E) Cell viability was measured at the indicated times. The results are presented as a percentage of the time-matched each CHI3L1 or control shRNA transuded cells in normoxia culture. (F) BEAS-2B cells transfected with a CHI3L1-overexpressing or empty vector were exposed to hyperoxia for 48 h. CHI3L1, cleaved caspase-3, and cleaved caspase-7 levels were determined by Western blot analysis. The data in A, C, and E represent the mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with time-matched controls. The data in B, D, and F are representative of at least three independent experiments.

contradictory results highlight the differences between the *in vivo* and *in vitro* effects of BRP-39/CHI3L1 on a cellular and systemic level. These results may also suggest different roles for CHI3L1 depending on the cell type. It should be considered that BEAS-2B is transformed human airway epithelial cells which are different from primary airway epithelial cells or alveolar type II epithelial cells. Additional studies are required to elucidate the differential role of CHI3L1 and expand our understanding.

In this study, we also explored the signaling pathways through which CHI3L1 performs its regulatory role in hyperoxia-exposed BEAS-2B cells. The predominant MAPK family members are ERK1/2, JNK, and p38 [28]. JNK and p38 are often implicated in the induction of cell death and inflammation after exposure to a variety of stimuli. However, ERK1/2, along with PI3K-Akt, is generally considered a cell survival-promoting signaling pathway that mediates cell proliferation [29,30]. Previous studies have illustrated that MAPKs and PI3K play critical roles in the regulation of hyperoxia-induced epithelial cell death [29–31]. *In vitro* assays with connective tissue cells demonstrated the ability of CHI3L1 to activate the MAPK and PKB/Akt pathways [32]. Based on these studies, we hypothesized that CHI3L1 may also regulate hyperoxia-induced cell death via the MAPK and PI3K pathways. Our results showed that exposure to hyperoxia enhanced ERK1/2 and p38 phosphorylation but reduced Akt activation. In the absence of CHI3L1, ERK1/2 and Akt phosphorylation were significantly increased while p38 activation was reduced. Consistent with the

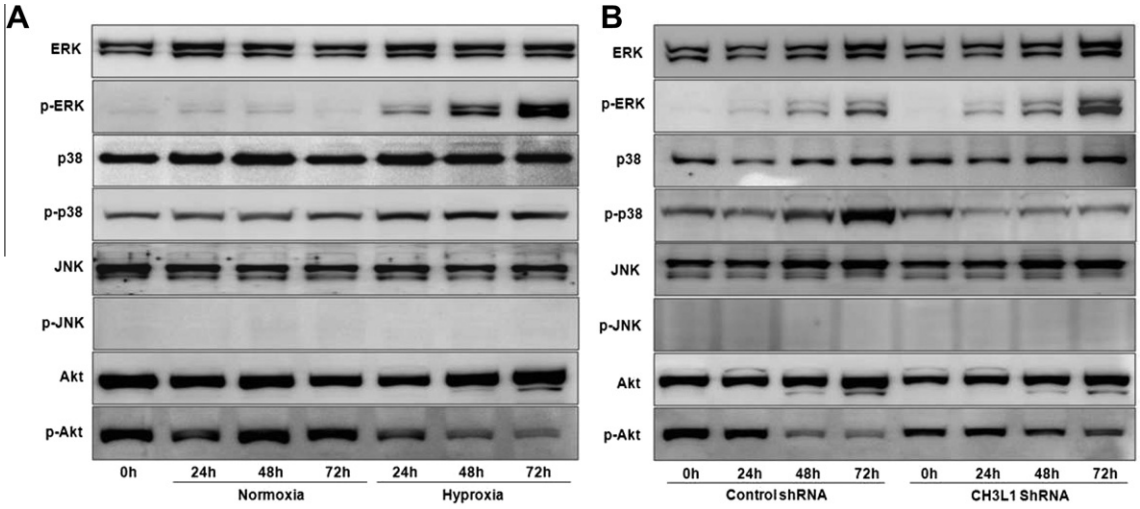

Fig. 4. MAPK and PI3K signaling pathways participate in CHI3L1 function. (A) BEAS-2B cells were exposed to either hyperoxia or normoxia for 24, 48, and 72 h. The cells were then harvested for Western blot analysis of ERK1/2, JNK, p38, and Akt phosphorylation. (B) Cells transfected with either CHI3L1 shRNA or control shRNA were exposed to hyperoxia for the indicated times. ERK1/2, JNK, p38, and Akt phosphorylation were determined by Western blot analysis. All results represent at least three independent experiments.

general role of MAPKs and PI3K, our results suggest that CHI3L1 mediates its regulatory function by regulating the phosphorylation status of the MAPK and PI3K signal transduction pathways. Chen et al. also demonstrated that the antioxidant thioredoxin prevents hyperoxia-induced apoptosis in fetal type II epithelial cells by downregulating JNK and p38 phosphorylation while upregulating ERK1/2 and Akt activation [33]. Based on these results, the authors concluded that both ERK1/2 and PI3K activation are required for the cytoprotective effect in lung epithelial cells.

In conclusion, our study investigated the regulatory function of CHI3L1 in airway epithelial cell death after hyperoxia exposure. Our results demonstrate that CHI3L1 accomplishes its function at least in part by affecting the activation of caspase-3 and -7. Moreover, we showed that the regulatory role of CHI3L1 is mediated via ERK1/2, p38, and Akt. Our findings suggest that CHI3L1 may be one of several cell death regulators that regulate the MAPK and PI3K signaling pathways following oxidative stress in human airway epithelial cells.

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