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# The protective effects of glutamine against bronchopulmonary dysplasia are associated with MKP-1/MAPK/cPLA2 signaling-mediated NF-kappaB pathway

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**Abstract.** Glutamine is proven to have potential therapeutic effects on decreasing hyperoxia-induced acute pulmonary injury. The aim of this study is to investigate the effects and mechanism of glutamine on bronchopulmonary dysplasia (BPD) induced by hyperoxia in rat alveolar type II epithelial cells (AECIIs) RLE-6TN. Following hyperoxia induction and glutamine treatment, ROS levels were detected by DCFH-DA assay and TUNEL staining was performed to detect cell apoptosis. The levels of inflammatory indicators and expression of apoptosis-related proteins were detected through ELISA and Western blot, respectively. Besides, the expression of related proteins in mitogen-activated protein kinase phosphatase-1 (MKP-1)/mitogen-activated protein kinases (MAPK)/cytoplasmic phospholipase A2 (cPLA2) signaling was also detected by Western blot. To further analyze the role of MKP-1/MAPK/cPLA2 signaling, MKP-1 was silenced and anisomycin was used to treat cells, respectively. It was shown that glutamine significantly decreased inflammation, oxidative stress and apoptosis in hyperoxia-induced cells while MKP-1 interference and anisomycin were able to reverse these effects, suggesting that the protective effects of glutamine on BPD induced by hypoxia were related to MKP-1/MAPK/cPLA2 signaling. To sum up, glutamine protected against BPD by decreasing inflammation, oxidative stress and apoptosis *via* MKP-1/MAPK/cPLA2 signaling.

**Key words:** Bronchopulmonary dysplasia — Glutamine — Inflammation — Oxidative stress — Apoptosis — MKP-1/MAPK/cPLA2 signaling

## Introduction

Bronchopulmonary dysplasia (BPD) is a chronic inflammatory lung disease characterized by pulmonary arrest and hypoxia (Rivera et al. 2016). With the progress of clinical practice in neonatal intensive care unit and development of perinatal medicine, the life quality of premature infants has improved significantly, but BPD has become one of the most common complications after hyperoxia and mechanical ventilation treatment (Balasubramaniam et al. 2007).

Several studies have confirmed that alveolar type II epithelial cells (AECIIs) are key target cells for BPD alveolar dysplasia (Lu et al. 2011; Rock and Hogan 2011). Immature AECII cells enter the air from the intrauterine hypoxic environment, or under the stimulation of increasing oxygen concentration of auxiliary ventilation equipment, lead to decreased proliferation ability of AECIIs, aggravation of DNA damage and other oxidative stress injury, which are considered to be the key mechanism of alveolar development disorder (Roper et al. 2004; Rawlins 2011).

It was reported that glutamine, a conditionally essential amino acid, was able to improve hyperoxia-induced acute pulmonary injury in adult mice partly through decreasing systemic inflammatory cytokine level (Perng et al. 2010).

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A previous study found that glutamine could alleviate hyperoxia-induced BPD in rats and regulate mitogen-activated protein kinase phosphatase-1 (MKP-1)/mitogen-activated protein kinases (MAPK)/cytoplasmic phospholipase A2 (cPLA2) signaling pathway. MAPK/cPLA2 signal transduction pathway has multidirectional biological functions in the organism. MAPK is a class of serine/threonine protein kinases, which belong to the intersection of common pathways of cell proliferation and cell differentiation, including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and P38 kinases. P38 kinase is the classic pathway of inflammatory response (Lee et al. 2013). cPLA2 is widely present in cells of various tissues and promotes the generation of inflammatory mediators and a large number of oxygen free radicals (Nanda et al. 2007; Chuang et al. 2015). MKP-1 plays an important negative role in MAPK regulation, which is a classic member of the archetypal protein phosphatase family and can specifically dephosphorylate and inactivate P38, JNK and ERK to inhibit the biosynthesis of cytokines. In the process of dephosphorylation, MKP-1 has a higher affinity for JNK and P38 than ERK (Comalada et al. 2012).

Considering the protective role of glutamine in hyperoxia-induced acute pulmonary injury, the present study is performed to investigate the mechanism of action of glutamine in hyperoxia-stimulated RLE-6TN cells.

## Materials and Methods

### *Cell culture*

The rat alveolar type II cell line (RLE-6TN) was purchased from Ningbo Mingzhou Biotechnology Co., LTD (Ningbo, China) and cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum with 5% $CO_2$ at 37°C. For hyperoxia model, 100% $CO_2$ and 100% $O_2$ were mixed in a 5:95 ratio with an air mixing apparatus. Cells were exposed to hyperoxia (inlet mixture gas contained $O_2$ (950 ml/l) and $CO_2$ (50 ml/l) at a speed of 3 l/min for 10 minutes) and then cultured in a humidified incubator (5% $CO_2$, 37°C) for 24 h.

### *CCK8 assay*

RLE-6TN cells were seeded into 96-well plate and the cell viability was detected by CCK8 kit according to the manufacturer's protocol (Beyotime, Shanghai, China). The absorbance was detected using BIO-RAD microplate reader (USA).

### *DCFH-DA assay*

The ROS levels were evaluated through DCFH-DA assay (Reactive oxygen species assay kit, Beyotime, Shanghai, China). DCFH-DA does not fluoresce itself, but can pass through the cell membrane freely and enter the cells. DCFH-DA can be hydrolyzed by intracellular esterase to generate DCFH. However, DCFH does not penetrate the cell membrane, so the probe is easily accumulated in the cells. ROS in the cells can oxidize the non-fluorescent DCFH to generate fluorescent DCF. The green fluorescence intensity was proportional to the level of ROS. The cells were observed under a fluorescence microscope (Thermo Fisher).

### *The detection of MDA and SOD*

The cells were collected and malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were detected using corresponding kits according to the manufacturer's guidance (Lipid Peroxidation (MDA) Assay kit, Superoxide Dismutase Activity Assay kit, Abcam, England).

### *ELISA assay*

The collected cells were used to detect the levels of tumor necrosis factor alpha (TNFα), interleukin-1beta (IL-1β) and interleukin-6 (IL-6) through ELISA kits following the manufacturer's protocol (Beyotime. Shanghai, China).

### *RT-qPCR assay*

Total RNA of collected cells was extracted using Trizol method. The synthesis of complementary DNA (cDNA) was performed using cDNA kit in accordance with the manufacturer's protocol (Thermofisher). Following the manufacturer's guidance, its amplification was performed using RT-qPCR kit (Thermofisher). The mRNA levels were detected using $2^{-\Delta\Delta Ct}$ method.

### *Western blot assay*

Total protein was extracted from RIPA lysate containing protease inhibitor and the concentration was determined by BCA method. Following separation using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were blocked using 50 g/l skim milk for 1 h at room temperature and then washed with PBST for three times. Subsequently, the membranes were incubated with the primary antibodies (abcam, England) overnight at 4°C and then incubated with secondary antibodies (abcam, England). The absorbance (A) value of each band was analyzed by ImageJ software and the relative expression of the target protein was expressed by the ratio of the A value of the target protein to the A value of the internal reference.

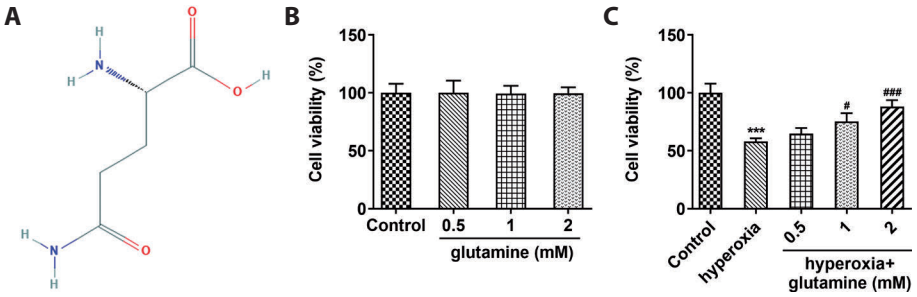



**Figure 1.** Glutamine increases cell viability in hyperoxia-induced RLE-6TN cells. **A.** Chemical structure of glutamine. Cell viability of each group after glutamine treatment (**B**) or cotreatment of glutamine and hyperoxia (**C**). *** $p < 0.001$ *vs.* control; # $p < 0.05$, ### $p < 0.001$ *vs.* hyperoxia.

*TUNEL assay*

The apoptosis of RLE-6TN cells were evaluated through TUNEL kit (Roche). Under the microscope, apoptotic cells showed green, while nuclei showed blue in DAPI staining. The percentage of TUNEL positive cells was calculated using the formula: apoptosis index = apoptotic cell number/total cell number.

*Plasmid transfection*

siRNA targeting MKP-1 or its negative control (siRNA-NC, scrambled RNA) were transfected into RLE-6TN cells by Lipofectamine 3000 in accordance with the manufacturer's guidance (Thermofisher). After transfection for 24 h, the cells were collected for further experiments.

*Statistical analysis*

The experimental data were shown in mean ± standard deviation (SD). GraphPad Prism 8.0 software was used to determine the difference among multiple groups by one-way analysis of variance, followed by Tukey's test. $p < 0.05$ mean that the difference was statistically significant.

## Results

*Glutamine increases cell viability and ameliorates oxidative stress in hyperoxia-induced RLE-6TN cells*

To determine the effects of glutamine, RLE-6TN cells were treated with different concentrations of glutamine and cell viability was measured through CCK8 assay. The 2D structure of glutamine is shown in Figure 1A. There were no significant changes in cell viability after glutamine treatment when compared with control group (Fig. 1B). Furthermore, the cell viability was higher in the group with treatment of hyperoxia and glutamine than that in hyperoxia group (Fig. 1C). DCFH-DA staining was performed to evaluate ROS levels. Among the hyperoxia-induced groups, ROS levels in glutamine-treated group were significantly decreased in a dose-dependent manner compared with control group (Fig. 2A). To evaluate oxidative stress, MDA content and SOD activity were measured. MDA content was decreased and SOD activity was increased when cells were co-treated with glutamine and hyperoxia compared with the group treated by hyperoxia alone (Fig. 2B,C).

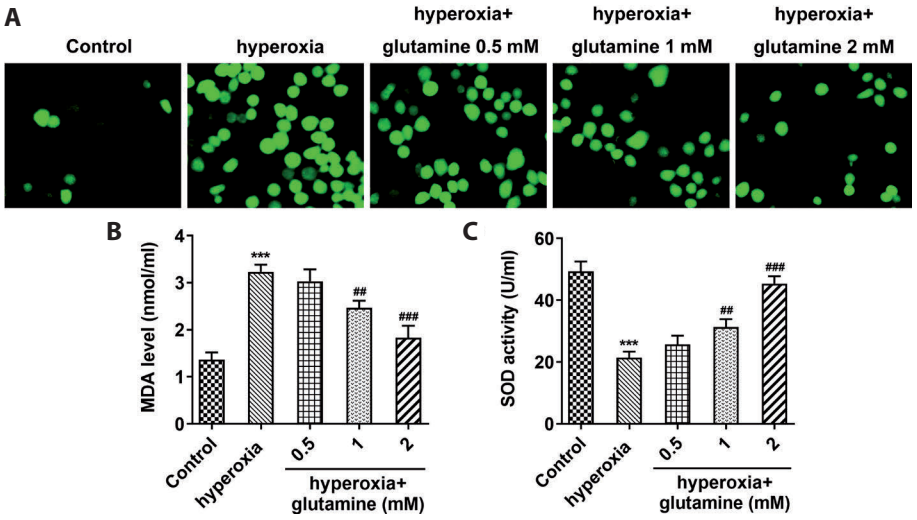



**Figure 2.** Glutamine ameliorates oxidative stress in hyperoxia-induced RLE-6TN cells. **A.** Representative images of DCFH-DA staining for the analysis of ROS levels. **B.** MDA content. **C.** SOD activity. *** $p < 0.001$ *vs.* control; ## $p < 0.01$, ### $p < 0.001$ *vs.* hyperoxia.

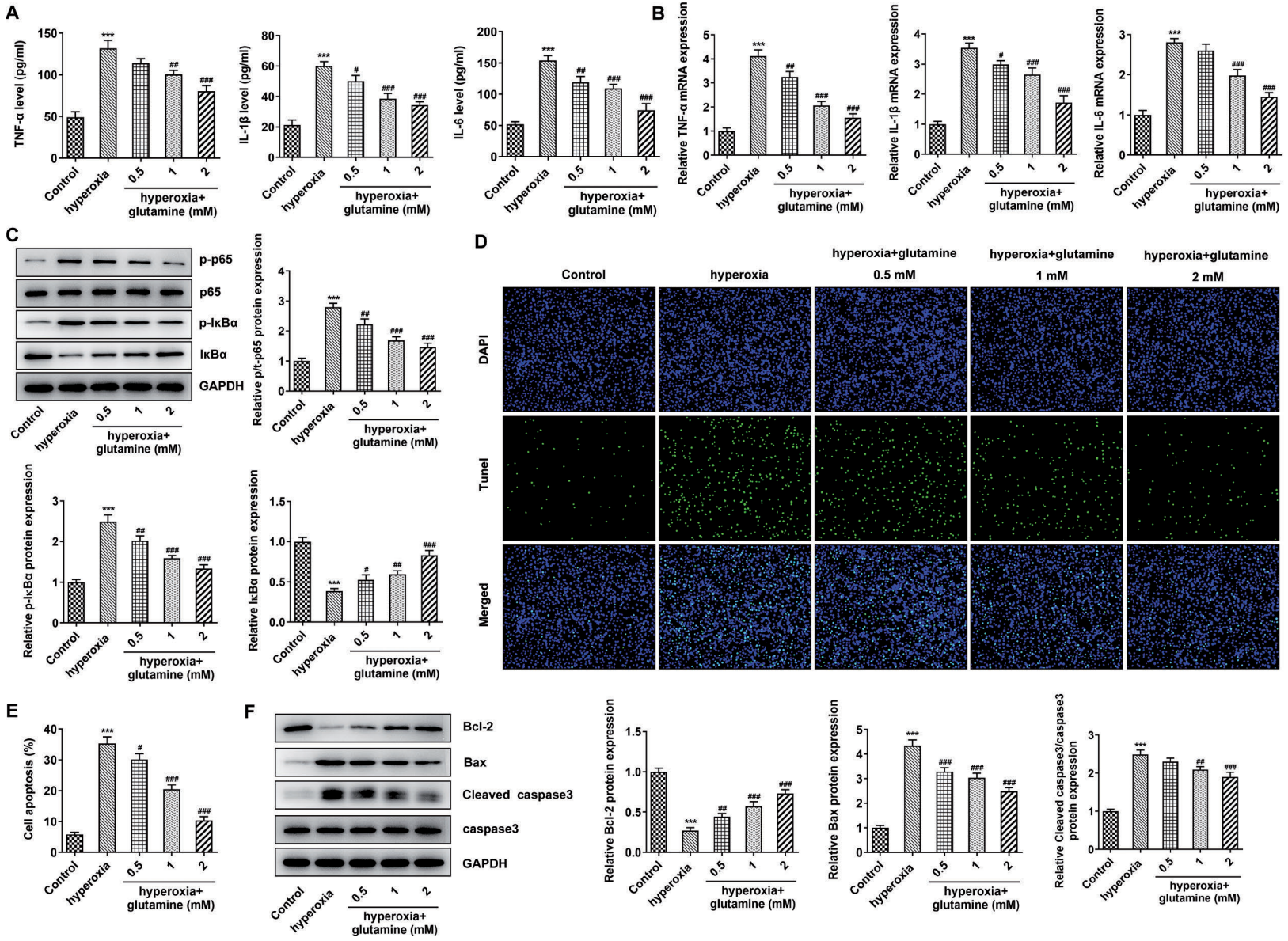



**Figure 3.** Glutamine alleviates hyperoxia-induced inflammatory response and apoptosis of RLE-6TN cells. The levels of TNFα, IL-1β and IL-6 through ELISA (**A**) and RT-qPCR (**B**) analysis. **C.** Western blot analysis of protein levels of p-p65, p-IκBα, p65 and IκBα. **D, E.** Representative images of TUNEL staining for cell apoptosis and analysis. **F.** The protein levels of BCL2, Bax and cleaved caspase3 through Western blot analysis. *** $p < 0.001$ *vs.* control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs.* hyperoxia.

*Glutamine alleviates hyperoxia-induced inflammatory response and apoptosis of RLE-6TN cells*

Through analysis of ELISA and RT-qPCR, hyperoxia increased the levels of TNFα, IL-1β and IL-6 compared with control group while glutamine was able to ameliorate the impact of hyperoxia on the levels of these inflammatory factors (Fig. 3A,B). In Western blot analysis, the protein levels of p-p65, p-IκBα, p65 and IκBα were detected. Among the hyperoxia groups, the protein levels of p-p65 and p-IκBα were decreased in glutamine-treated group (Fig. 3C). In addition, hyperoxia significantly induced higher apoptotic levels compared with that in control group. However, the apoptotic levels in glutamine-treated group in RLE-6TN cells under the condition of hyperoxia were markedly improved when compared with hyperoxia group (Fig. 3D,E). Decreased Bcl-2 protein level and increased protein levels of Bax and cleaved caspase3 were also found in hyperoxia group without glutamine treatment (Fig. 3F).

*Glutamine modulates MKP-1/MAPK/cPLA2 signaling in hyperoxia-treated RLE-6TN cells*

To determine the effects of glutamine on MKP-1/MAPK/cPLA2 signaling, Western blot analysis of the expression of related factors in MKP-1/MAPK/cPLA2 signaling was performed in RLE-6TN cells. The cells exposed to hyperoxia showed decreased MKP-1 expression and increased phosphorylated levels of p38 and cPLA2. However, glutamine treatment could significantly reverse the influences imposed by hyperoxia induction in a dose-dependent manner (Fig. 4). MKP-1 regulates the extent and duration of pro-inflammatory MAPK signaling in the airway and has anti-apoptotic and anti-inflammatory effects by simultaneously controlling the activity of JNK and microglia P38 (Liu et al. 2014; Moosavi et al. 2017). To evaluate the role of MKP-1/MAPK/cPLA2 signaling, the siRNAs silencing MKP-1 was constructed. Both siRNA-MKP-1-1 and siRNA-MKP-1-2 exhibited significant inhibitory effects on MKP-1 expression (Fig. 5A,B). The p-p38 and p-cPLA2 protein levels were markedly increased in siRNA-MKP-1 group when compared with siRNA-NC group (Fig. 5C). To further evaluate the role of MKP-1/MAPK/cPLA2 signaling, anisomycin, a MAPK agonist, was used to treat cells. Following treatment of siRNA-MKP-1 or anisomycin in RLE-6TN cells co-treated by hyperoxia and glutamine, cell viability was evaluated by CCK8 assay. MKP-1 silencing or anisomycin treatment markedly counteracted the effects of glutamine on cell viability in hyperoxia-induced cells (Fig. 5D,E). To further evaluate oxidative stress levels, the levels of ROS and MDA, and SOD activity were analyzed. MKP-1 silencing or anisomycin treatment significantly reduced levels of ROS and MDA, and SOD activity in the hyperoxia and glutamine-co-treated cells (Fig. 5F–K). These results suggested that glutamine modulates MKP-1/MAPK/cPLA2 signaling to increase cell viability and decrease oxidative stress in hyperoxia-induced cells.

*Glutamine modulates MKP-1/MAPK/cPLA2 signaling to ameliorate inflammation and apoptosis in hyperoxia-treated RLE-6TN cells*

Inflammation and apoptosis were analyzed to evaluate the role of MKP-1/MAPK/cPLA2 signaling involved in the effects of glutamine on hyperoxia-insulted RLE-6TN cells. We detected the levels of TNFα, IL-1β and IL-6 when both hyperoxia and glutamine-cotreated cells were also transfected with MKP-1 interference plasmids or subjected to anisomycin treatment. MKP-1 silencing or anisomycin treatment restored the significant effects of glutamine on reducing the increased levels of TNFα, IL-1β and IL-6 under hyperoxia stimulation (Fig. 6A–D). Additionally, MKP-1 silencing or anisomycin treatment increased the phosphorylation of p65 and IκBα in hyperoxia and glutamine-cotreated cells (Fig. 6E,F). TUNEL staining showed that MKP-1 silencing or anisomycin treatment promoted apoptosis, whereas they decreased Bcl-2 levels and increased the protein levels of Bax and cleaved caspase3 in RLE-6TN cells which received the co-treatment of hyperoxia and glutamine (Fig. 6G–J).

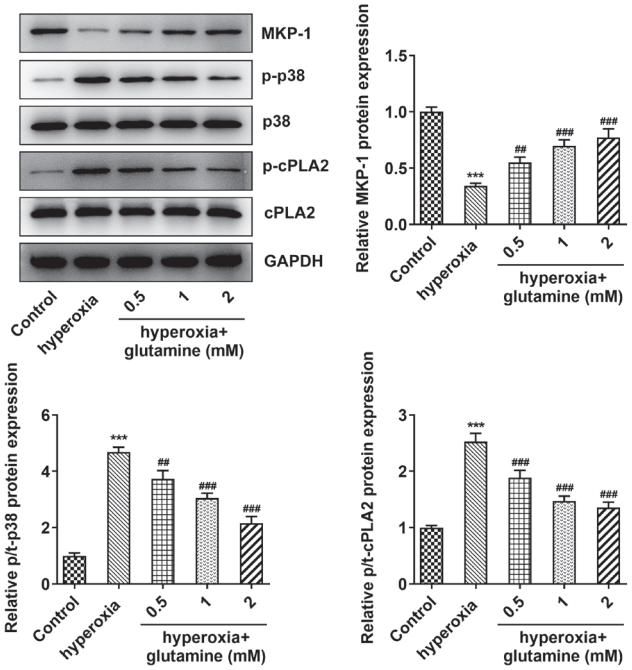



**Figure 4.** Glutamine modulates MKP-1/MAPK/cPLA2 signaling in hyperoxia-treated RLE-6TN cells. Western blot analysis of protein levels of related factors in MKP-1/MAPK/cPLA2 signaling. *** $p <$ 0.001 *vs*. control; ## $p < 0.01$, ### $p < 0.001$ *vs*. hyperoxia.

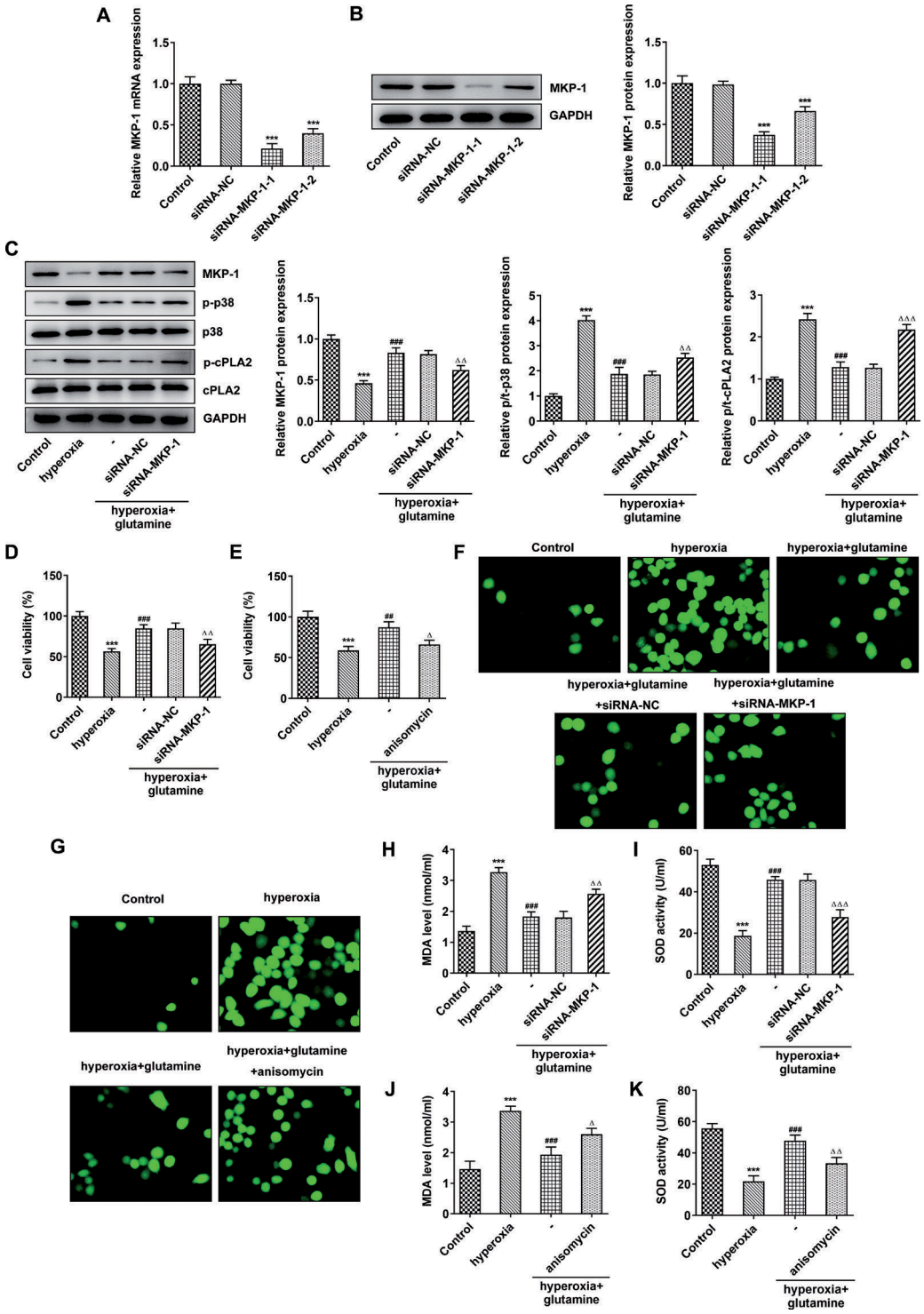
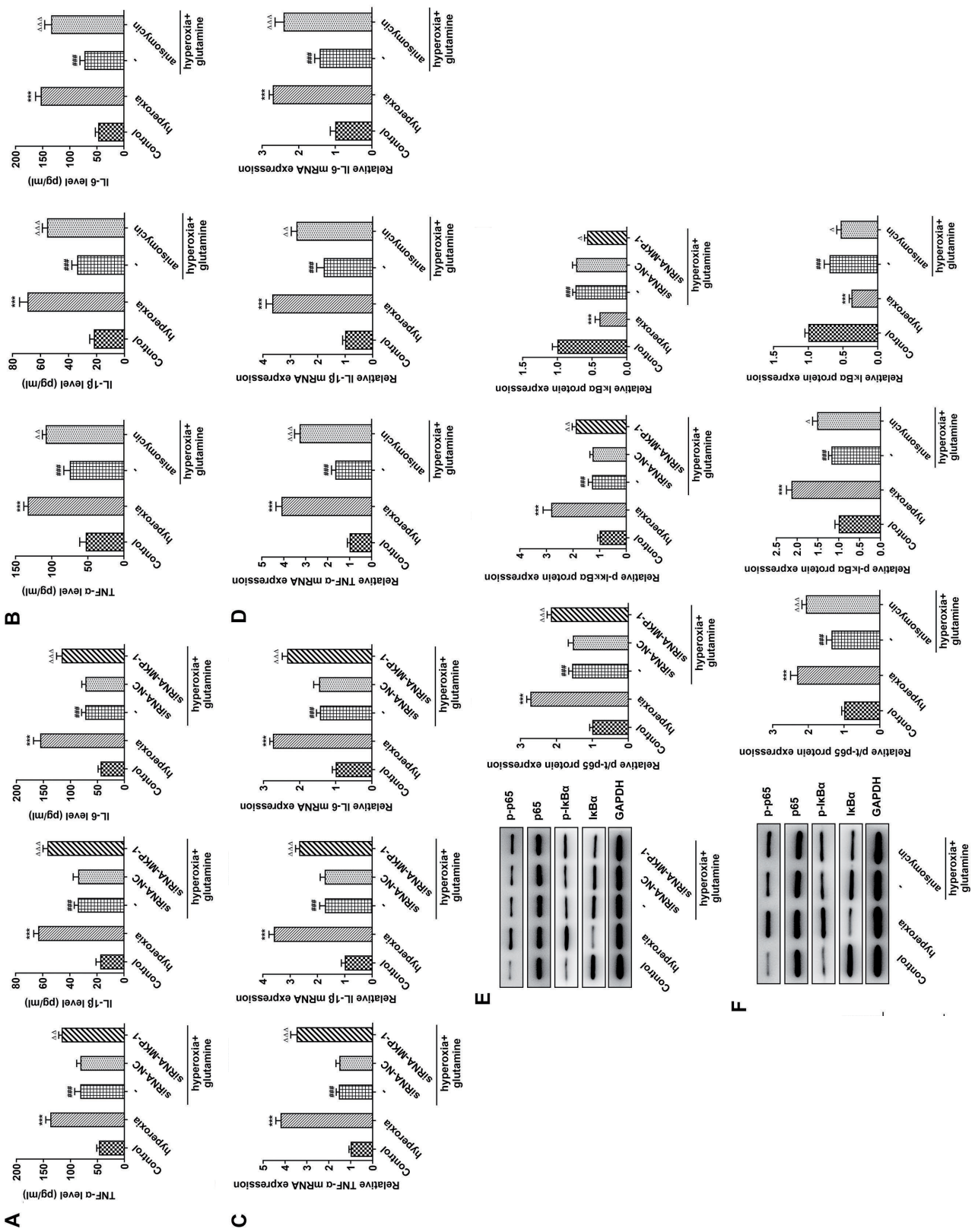



**Figure 5.** Glutamine modulates MKP-1/MAPK/cPLA2 signaling to increase cell viability and ameliorates oxidative stress in hyperoxia-treated RLE-6TN cells, **A, B.** The knockdown efficacy of siRNA-MKP-1-1/2 plasmids. *** $p < 0.001$ *vs.* siRNA-NC. **C.** Western blot analysis of protein levels of related factors in MKP-1/MAPK/cPLA2 signaling. **D.** CCK8 assay for detection of cell viability when MKP-1 was silenced. **E.** CCK8 assay for detection of cell viability after anisomycin addition. **F.** Representative images of DCFH-DA staining when MKP-1 was silenced. **G.** Representative images of DCFH-DA staining after anisomycin addition. **H, I.** MDA levels and SOD activity when MKP-1 was silenced. **J, K.** MDA levels and SOD activity after anisomycin addition. *** $p < 0.001$ *vs.* control; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ *vs.* hyperoxia; $^{\Delta}$ $p < 0.001$, $^{\Delta\Delta}$ $p < 0.01$, $^{\Delta\Delta\Delta}$ $p < 0.001$ *vs.* hyperoxia+glutamine+siRNA-NC.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Relative TNF-α mRNA expression
Relative IL-1β mRNA expression
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Relative TNF-α mRNA expression
Relative IL-1β mRNA expression
Relative IL-6 mRNA expression
Relative p-IκBα protein expression
Relative IκBα protein expression
Relative p/t-p65 protein expression
Relative p-IκBα protein expression
Relative IκBα protein expression
Relative p/t-p65 protein expression
p-p65
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IκBα
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siRNA-NC
siRNA-MKP-1
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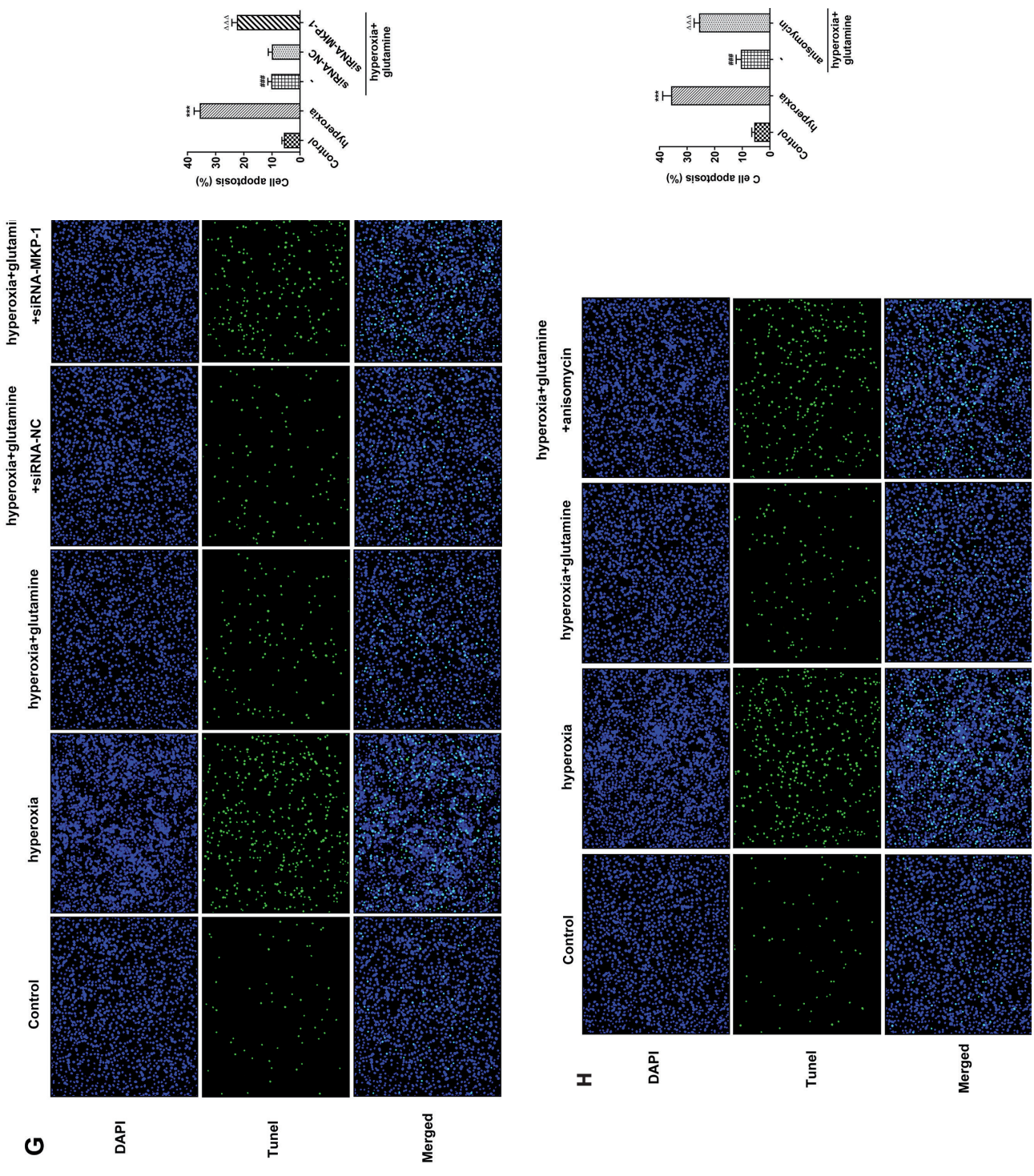
hyperoxia+ glutamine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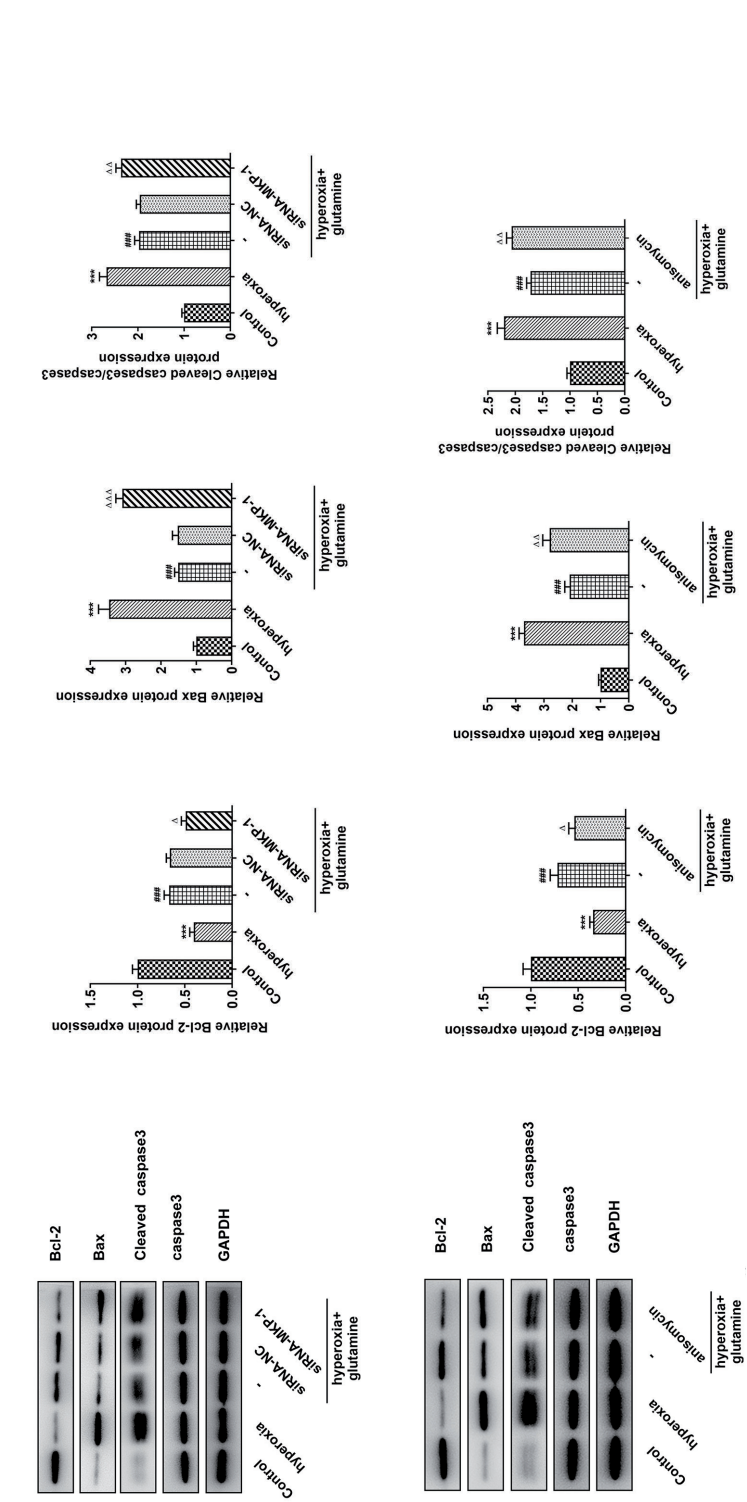


**Figure 6.** Glutamine modulates MKP-1/MAPK/cPLA2 signaling to ameliorate inflammation and apoptosis in hyperoxia-treated RLE-6TN cells. **A.** The levels of TNFα, IL-1β and IL-6 by ELISA assay when MKP-1 was silenced. **B.** The levels of TNFα, IL-1β and IL-6 by ELISA assay after anisomycin addition. **C.** The levels of TNFα, IL-1β and IL-6 by RT-qPCR assay when MKP-1 was silenced. **D.** The levels of TNFα, IL-1β and IL-6 by RT-qPCR assay after anisomycin addition. **E.** Differential protein levels of p-p65, p-IκBα, p65 and IκBα when MKP-1 was silenced. **F.** Differential protein levels of p-p65, p-IκBα, p65 and IκBα after anisomycin addition. **G.** Representative images of TUNEL staining when MKP-1 was silenced. **H.** Representative images of TUNEL staining after anisomycin addition. **I.** Bcl-2 protein levels and the protein levels of Bax and cleaved caspase3 through Western blot detection when MKP-1 was silenced. **J.** Bcl-2 protein levels and the protein levels of Bax and cleaved caspase3 through Western blot detection after anisomycin addition. *** $p < 0.001$ *vs.* control; ### $p < 0.001$ *vs.* hyperoxia; $^{\Delta}$ $p < 0.001$, $^{\Delta\Delta}$ $p < 0.01$, $^{\Delta\Delta\Delta}$ $p < 0.001$ *vs.* hyperoxia+glutamine+siRNA-NC.

## Discussion

In the present study, we demonstrated that glutamine had a protective effect on hyperoxia-induced cell injury by decreasing oxidative stress, inflammation and apoptosis by MKP-1/MAPK/cPLA2 signaling. The most important finding of our work was the significant alleviation of oxidative stress, inflammation and apoptosis in hyperoxia-induced alveolar type II cells after glutamine treatment compared with hyperoxia group. The possible mechanisms underlying this beneficial effect could be associated with the inhibition of MKP-1/MAPK/cPLA2 signaling under glutamine treatment. We further observed that the effects of glutamine on oxidative stress, inflammation and apoptosis were blocked by MKP-1 silencing or anisomycin. These results revealed that oxidative stress, inflammation and apoptosis induced by hyperoxia were suppressed by glutamine treatment and MKP-1/MAPK/cPLA2 signaling was involved in this process.

A large number of clinical evidences have found that high concentrations of oxidative stress products, such as lipid hydrogen peroxide and MDA, can be found in plasmas or bronchoalveolar lavage fluid samples of premature infants who eventually develop BPD in the first 24 to 48 hours after birth. It is suggested that oxidative stress-evoked lung injury is an important cause of BPD (Pan et al. 2009; Fabiano et al. 2016). The present study showed the anti-oxidation effect of glutamine and indicated the potential protective effects of glutamine on improving BPD.

There are a growing number of studies revealing the anti-inflammation and apoptosis effects of glutamine (Li et al. 2017; Cheng et al. 2021; Liu et al. 2021). In the present study, we detected inflammation and apoptosis and also found that glutamine reduced the levels of TNFα, IL-1β, and IL-6, and suppressed apoptosis. Additionally, glutamine treatment increased the phosphorylation of p65 and IκBα, suggesting that the decreased levels of TNFα, IL-1β and IL-6 were possibly related to glutamine-regulated NF-κB pathway in hyperoxia-treated AECIIs cells. According to previous studies, glutamine exerts protective effects on improving lung injury, which is partly related to NF-κB pathway (Singleton and Wischmeyer 2007; Kwon et al. 2010; Peng et al. 2011). In our work, glutamine treatment upregulated MKP-1 expression and reduced the phosphorylation levels of p38 and p-cPLA2. According to a previous report, glutamine displays anti-inflammatory activity *via* MKP-1 induction dependent on ERK (Ayush et al. 2016).

## Conclusion

In conclusion, we demonstrated that glutamine improved hyperoxia-induced AECIIs injury, which was revealed to be associated with the inhibition of oxidative stress, inflammation and apoptosis. Furthermore, the effects of glutamine were likely to be mediated by MKP-1/MAPK/cPLA2 signaling. Therefore, glutamine might be a potential therapy for BPD.

**Availability of data and materials.** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**Ethical statement.** The study doesn't involve animal and clinical samples.

**Conflict of interests.** The authors declare that they have no competing interests.

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