ORIGINAL RESEARCH

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Moderate hyperoxia plays a protective role in lung bronchial epithelial cells

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Abstract

Purpose: Oxygen therapy is commonly used in clinical settings, but several problems may result from improper use. Oxygen poisoning involves the initiation of a series of inflammatory reactions. In this study, we compared the effects of moderate hyperoxia (40% O₂) and extreme hyperoxia (85% O₂) on pulmonary bronchial epithelial cells.

Methods: Normal human tracheobronchial epithelium (NHBE) cells were exposed to hyperoxia (40% and 85%) for 24 hours, and their survival rates were determined by the colorimetric assay, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The protein and mRNA levels of RelA, RelB, ASK1, TNF- α and secretory component (SC) were detected by immunohistochemical staining, western blot, and real-time polymerase chain reaction.

Results: The NHBE cell survival increased in the presence of moderate hyperoxia. RelA, RelB, ASK1, TNF- α and SC expressions were significantly higher in the 85% O₂ group in comparison with the control group and the 40% O₂ group. In the 40% O₂ group, RelA, RelB, ASK1 and TNF- α were up-regulated, but SC expression was not significantly different than that of the control group. However, compared with the 85% O₂ group, SC expression was significantly lower in the 40% O₂ group.

Conclusion: These results suggest that moderate hyperoxia promotes proliferation in NHBE cells and activates TNF- α and downstream ASK1. Then TNF- α activates NF- κ B and SC to play a protective role.

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The purpose of oxygen therapy is to ensure an adequate supply of oxygen to the tissues and to eliminate or reduce disease-related adverse effects. Although oxygen is the one of the most common interventions used in the clinic, improper oxygen therapy can also cause several problems. Oxygen poisoning can directly damage alveolar epithelial cells, initiating a series of inflammatory reactions.

The cytokine tumor necrosis factor- α (TNF- α) is expressed in several cell types and regulates apoptosis and necrosis [1]. The TNF- α is a major inflammatory cytokine that plays an important role in a number of chronic and acute lung diseases, including hyperoxia-induced lung damage [2,3]. The TNF- α interacts with TNF receptors and promotes TNF receptor-associated factors to activate apoptosis signal-regulating kinase-1 (ASK1). Activation of ASK1 initiates apoptosis in endothelial cells and perpetuates reactive oxygen species-induced (ROS) inflammatory responses [4,5]. Nuclear factor- κ B (NF- κ B) is a key transcription factor involved in the synthesis of TNF- α , and also regulates oxidative stress-sensitive transcription. The transcription factors RelA, RelB, REL, NF- κ B1 (p50) and NF- κ B2 (p52), are all members of the NF- κ B family. Two distinct pathways, called the classical and non-classical pathways, lead to the activation of NF- κ B dimers [6]. Dysregulation of NF- κ B has been associated with various pathological conditions, including hyperoxia [7]. Additionally, TNF- α can promote the secretion of secretory component (SC), a component of the mucosal immune system that plays a protective role during hypoxia-induced acute lung injury [8,9].

Although many studies have examined the adverse effects of hyperoxia *in vitro* and *in vivo* [10], most have focused on extreme hyperoxia (oxygen concentrations > 80%) [11-13]. In comparison, little is known about the effects of moderate hyperoxia (oxygen concentrations 40%–60%) on the lungs [14], despite the frequent use of 40% oxygen for the treatment of many pulmonary diseases [15]. In this study, we compared the effects of moderate hyperoxia (40% O₂) and extreme hyperoxia (85% O₂) on lung bronchial epithelial cells to provide a theoretical basis for clinical oxygen therapy use.

Materials and methods

Cell culture

Normal human tracheobronchial epithelium (NHBE) cells were obtained from the Cell Biological Institute of Shanghai, Chinese Academy of Sciences. The cells were cultured in sterile basal medium/DMEM-H (Sigma; St. Louis MO, USA) containing 10% fetal bovine serum (FBS, Genview, Beijing,

China), 100 U penicillin/ml, 100 mg streptomycin/ml, 1% L-glutamine, and 0.25 mg amphotericin B/ml, and were incubated at 37°C with 5% CO₂. Cells (1×10⁶ cells/ml) were plated and cultured for 24 hours and were then exposed to moderate (40% O₂) or extreme hyperoxia (85% O₂) (CB160, BINDER, Germany) for 24 hours. The control cells were not exposed to hyperoxia. Protein and mRNA were subsequently extracted from the cells. Each experiment was repeated 6–8 times.

MTT assay

Prior to treatment, the cells were plated onto 96-well microtiter plates with fresh medium and cultured for 24 hours. The cells were then treated with 40% or 85% O₂ for 24 hours. Cells which were not exposed to hyperoxia were used as a control. Next, the cells were treated with 20 μ L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37°C. The MTT reaction was stopped by adding dimethyl sulfoxide (DMSO). The absorbance of each well at 490 nm was determined. Each experiment was repeated six times.

Immunohistochemical analysis

Cells were placed on coverslips and treated with 40% or 85% O₂ for 24 hours and then fixed with 4% paraformaldehyde. Next, the cells were treated with 10% goat serum for 30 minutes and then incubated with mouse anti-human RelA (Sigma), rabbit anti-human RelB (Sigma), and rabbit anti-human SC (Abcam, Cambridge, UK) overnight at 4°C, followed by an incubation with a secondary antibody (ZSGB-BIO, Beijing, China) for 40 minutes at 37°C. Finally, the cells were stained with diaminobenzidine (DAB) and counterstained with hematoxylin. Negative controls were created by replacing the primary antibody with phosphate-buffered saline. The median absorbance values of RelA, RelB and SC were determined using image analysis software (Prism, Shanghai, China) after scanning.

Western blot analysis

Whole protein extracts (40 ng) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and they were then transferred to polyvinylidene fluoride membranes. The membranes were then blocked with Tris-buffered-saline-Tween, containing 5% skim milk, at room temperature for 2 hours, and then incubated with mouse anti-human RelA (1:1000, Sigma), rabbit anti-human RelB (1:1000, Sigma), rabbit anti-human ASK1 (1:1000, Sigma), rabbit anti-human TNF α (1:1000, Abcam), rabbit anti-human SC (1:1000, Abcam) and GAPDH (1:10000, Kangchen, Shanghai, China)

overnight at 4°C. The membranes were then incubated with the appropriate secondary antibodies (1:5000, ZSGB-Bio, Beijing, China) and visualized using a super-enhanced chemiluminescence substrate and exposed to C300 (Azure Biosystems, CA, USA). The images were scanned, and a densitometry analysis of RelA, RelB, ASK1, TNF α and SC protein levels normalized to GAPDH was performed (Image-Pro Plus 6.0; Media Cybernetics).

Real-time polymerase chain reaction analysis

Total RNA was isolated from NHBE cells using RNA Mini Kits (RR047A, Takara Biotechnology, Dalian, China). The cDNA was prepared from 100 ng RNA (RR420A, Takara Biotechnology Co., Dalian, China). The polymerase chain reaction (PCR) amplification was performed using DNA polymerase for a preliminary cycle at 95°C for 10 seconds, followed by 45 cycles of 95°C for 5 seconds and 60°C for 20 seconds, followed by 1 minute at 60°C and 5 seconds at 95°C. Quantitative real-time PCR was performed using the LightCycler® 480 Real-TimePCR System (Roche Applied Science, Germany). The primers, fluorescent probes and the internal reference (GAPDH) were purchased from Takara Biotechnology. The primers for RelA, RelB, ASK1, TNF- α , and SC used in this study were as follows:

RelA-F: 5'- GGAGCACAGATACCACCAAGA-3';
 RelA-R: 5'- CGGCAGTCCCTTTCCTACAAG-3';
 RelB-F: 5'- TGTGGTGAGGATCTGCTTCCAG-3';
 RelB-R: 5'- GGCCCGCTTTCCTTGTTAATTC-3';
 ASK1-F: 5'- TTCACACAAAACGGATGTAACATT-3';
 ASK1-R: 5'-
 CCTAAACAGTTATGGTCACATTTTGG-3';
 TNF- α -F: 5'- GGCCTGGAGCTGAGAGATAA-3';
 TNF- α -R: 5'- GTGTGGGTGAGGAGCACAT-3';
 SC-F: 5'-GCTTGTCTCCCTGACCCTG-3';
 SC-R: 5'-AATGGCTTTGTTCTCAATCTC -3';
 GAPDH-F: 5'-GCACCGTCAAGGCTGAGAAC-3';
 GAPDH-R: 5'-TGGTGAAGACGCCAGTGGA-3'.

The efficiency of amplification for each target gene (GAPDH) was 100% in the exponential phase of PCR. The mRNA levels were normalized to GAPDH mRNA according to the following formula: active levels of target mRNA = $2^{-(CT\text{-target}-CT\text{-GAPDH})} \times 100\%$. The mRNA levels of cells exposed to different percentages of hyperoxia were compared with those in the control group.

Statistical analysis

All data are expressed as means $\pm$ standard deviations for each group. Multifactor variance analysis was used to

determine significant differences between each group. $P < 0.05$ was considered statistically significant.

Results

Moderate hyperoxia increased the survival rates of cells.

We first investigated the survival rates of NHBE cells in hyperoxic conditions. As shown in Figure 1, the survival rates in the control group were significantly lower than those in the 40% O₂ group ($P < 0.0001$) but significantly higher than those in the 85% O₂ group ($P < 0.0001$). These findings show that moderate hyperoxia promotes cell survival.

FIGURE 1. The survival rates of cells exposed to hyperoxia. Compared with the control group and the 85% O₂ group, the cell survival rates were significantly higher in the 40% O₂ group. Results are presented as means $\pm$ standard deviations. (**** $p < 0.0001$ compared with the control group; $n = 6$). Abbreviation: OD, optical density

Moderate hyperoxia stimulated TNF- α expression

Protein (Figures 2A, 2B) and mRNA expression levels of TNF- α were determined in the NHBE cells (Figure 2C). The protein and mRNA levels of TNF- α both increased with increasing concentrations of O₂ (Figures 2B, 2C). Compared with the control group, the TNF- α protein and mRNA levels increased significantly in the 40% and 85% O₂ groups ($P < 0.01$). Compared with the 85% O₂ group, the TNF- α protein and mRNA levels in the 40% O₂ were remarkably low ($P < 0.05$ or $P < 0.001$).

ASK1 expression was upregulated during moderate hyperoxia

Protein (Figure 2D-E) and mRNA (Figure 2F) expression levels of ASK1 were determined. Compared with the control group, ASK1 protein and mRNA expression increased in the

FIGURE 2. The influence of moderate hyperoxia on TNF- α and ASK1 expression. A and D) TNF- α (20 kDa) and ASK1 (135 kDa) protein expression. B and E) Densitometric analysis of TNF- α and ASK1 expression. C and F) mRNA levels of TNF- α and ASK1. The expression of TNF- α and ASK1 increased with increased oxygen concentration; however, TNF- α and ASK1 were lower in the 40% O₂ group than the 85% O₂ group. Results are presented as means $\pm$ standard deviations. (** p <0.01, *** p <0.0001 compared with the control group; # p <0.05, #### p <0.0001 compared with the 85% O₂ group; n=6).

40% and 85% O₂ groups (P <0.0001). Furthermore, ASK1 protein levels in the 40% O₂ group were lower than those in the 85% O₂ group (P <0.01). There was no significant difference in the ASK1 mRNA levels between the 40% O₂ and the 85% O₂ groups.

Moderate hyperoxia regulated the expression of RelA and RelB

RelA and RelB localizations within the cells were examined with immunohistochemical staining (Figure 3A, 3C). RelA and RelB were located mainly in the cytoplasm of the control cells; however, RelA and RelB were present in both the cytoplasm and nucleus in the 40% and 85% O₂ groups. The expressions of RelA and RelB increased in the 40% O₂ (RelA P <0.0001, RelB P <0.001) and 85% O₂ (P <0.0001) groups compared with the control group, but the expressions of RelA and RelB were lower in the 40% O₂

group compared with the 85% O₂ group (P <0.001, P <0.0001) (Fig. 3B and 3D).

Next, we detected the protein and mRNA levels of RelA and RelB. Compared with the control group, the levels of RelA and RelB were increased in the 40% O₂ group (P <0.01); however, the RelA protein level in the 40% group was significantly decreased compared with the 85% O₂ group (P <0.01). No significant difference in RelB protein levels was seen between the 40% and 85% O₂ groups (Figure 3E-G). RelA and RelB mRNA were significantly upregulated in the 40% O₂ group compared with the control group (P <0.05, P <0.001) (Figure 3H-J), but RelA mRNA was significantly downregulated in the 40% group compared with the 85% O₂ group (P <0.001) (Fig. 3H). No significant difference in the RelB mRNA level was seen between the 40% and 85% O₂ groups (Figure 3J).

FIGURE 3. Moderate hyperoxia regulated the expression of RelA and RelB. A and C) RelA and RelB staining, 400× magnification. B and D) The integrated optical density of RelA and RelB. E) RelA (65 kDa) and RelB (70 kDa) protein expressions. F and G) Densitometric analysis of RelA and RelB. H and J) The mRNA level of RelA and RelB. Compared with the control group, the levels of RelA and RelB were increased in the 40% O₂ group; however, the RelA in the 40% group was significantly decreased compared with the 85% O₂ group. No significant difference in RelB protein levels was seen between the 40% and 85% O₂ groups. The results are presented as means ± standard deviations (* p <0.05, ** p <0.05, *** p <0.001, **** p <0.0001 compared with the control group; ## p <0.01, ### p <0.001 compared with the 85% O₂ group; n=6).

Moderate hyperoxia affects the expression of SC

Immunohistochemical staining showed that SC was localized mainly in the cytoplasm (Figure 4A). There was no significant difference between the 40% O₂ and the control groups; however, SC expression was significantly higher in the 85% O₂ group compared with the 40% O₂ group and the control group ($P<0.0001$) (Figure 4B).

The SC protein analysis showed that there was no significant difference between the 40% O₂ and control

groups; however, SC protein expression was significantly lower in the 40% O₂ group compared with the 85% O₂ group ($P<0.0001$) (Figure 4C-D). The SC mRNA levels were increased in the 40% and 85% O₂ groups compared with the control group, but SC mRNA in the 40% O₂ group was significantly decreased compared with the 85% O₂ group ($P<0.001$) (Figure 4E).

FIGURE 4. Moderate hyperoxia influenced the expression of secretory component. A) SC staining, 400× magnification. B) Integrated optical density of SC. C) SC (85 kDa) protein expression. D) Densitometric analysis of SC expression. E) mRNA levels of SC. SC expression was increased in 85% O₂ group compared with the control group and 40% O₂ group. The results are presented as means $\pm$ standard deviations (** $p<0.001$, *** $p<0.0001$ compared with the control group; #### $p<0.0001$ compared with 85% O₂ group; $n=6$). Abbreviation: SC, secretory component

Discussion

The TNF- α can mediate a variety of acute and chronic lung diseases, including hyperoxia-induced lung injury [2,3]. Although the exact relationship between TNF- α and hyperoxia-induced lung injury remains unclear, it is known that TNF- α is involved in the early inflammatory response [16]. In our study, TNF- α expression in cells exposed to 40% O₂ was increased compared with control cells but was significantly lower compared with cells exposed 85% O₂. We also found that moderate hyperoxia promoted cell survival. These results indicate that moderate hyperoxia upregulates TNF- α (Figure 5), but to a lesser degree than extreme hyperoxia. It is possible that the lower TNF- α levels may stimulate the secretion of protective factors to increase cell survival.

FIGURE 5. Crosstalk of TNF- α , ASK1 and NF- κ B in hyperoxia.

During oxidative stress, TNF- α activates NF- κ B [17,18]. The ASK1 (Figure 5) activated by TNF- α results in destruction of the cell microtubule structures, leading to the accumulation of endoplasmic reticulum proteins and the transcription of genes in the nucleus. In resting cells, ASK1 forms an inactive complex with thioredoxin. When cells are exposed to H₂O₂ (as a reactive oxygen species (ROS) donor) or TNF- α , ASK1 dissociates from thioredoxin and becomes activated [19]. The ASK1 activation occurs when cells are exposed to various types of stress, including oxidative stress and inflammatory cytokines [20]. A recent study also showed that ASK1 was activated during hyperoxia-induced acute

lung injury, and ASK1 deletion protected against lung injury [21]. The ASK1 is involved in TNF- α -mediated apoptosis, which is induced by ROS. Additionally, ASK1 serves as a second messenger to correlate with diverse cellular processes, such as survival, differentiation and apoptosis [19]. The ASK1-deficient cells have decreased inflammatory responses resulting from the reduction of activation and production of proinflammatory cytokines such as TNF- α [22]. A previous study showed that ASK1 deletion *in vivo* significantly suppressed the hyperoxia-induced elevation of TNF- α and significantly inhibited the hyperoxic lung injury [21]. In our study, ASK1 expression was up-regulated at both the protein and mRNA levels in the 40% hyperoxia group compared with the control group. The expression of ASK1 in the 40% and 85% hyperoxia groups was not statistically different at the mRNA level, but at the protein level, ASK1 expression was significantly lower in the 40% versus the 85% hyperoxia group. Thus, moderate hyperoxia may cause transcription of ASK1 at similar levels as extreme hyperoxia, but there may be an unknown mechanism that down-regulates the translation of ASK1 during moderate hyperoxia, inhibiting the resultant inflammation.

The TNF- α is a major cytokine that can activate the NF- κ B pathway. Furthermore, feedback from NF- κ B activation results in an increase in TNF- α expression [17]. There are two pathways to activate NF- κ B: the classical pathway, which involves RelA; and the non-classical pathway, which involves RelB [23]. The TNF- α is associated with both pathways. In the classical pathway, the TNF- α receptor is recruited by membrane shuttle kinases, ultimately leading to the degradation of I κ B. The I κ B forms a complex with NF- κ B in the cytoplasm, and inhibits the nuclear localization of the dimer and activates RelA [24]. The TNF- α can induce the activation of I κ B α , resulting in nuclear translocation of RelA [25,26]. The TNF- α can also activate the non-classical NF- κ B pathway, resulting in p100 phosphorylation and activation of the RelB/p52 dimer [27]. However, a study by Etemadi et al. showed that activation of the non-classical pathway was likely to be independent of TNF because the levels of NF- κ B-inducing kinase and p52 did not change upon stimulation with TNF [28]. A study by Franek et al. showed that NF- κ B was active during hyperoxia for the first 24 hours but was quickly blunted after 72 hours [29]. This suggests that NF- κ B may play a protective role during short stints of hyperoxia, but the inflammatory response induced during prolonged hyperoxia eventually overcomes the protective response and leads to lung damage [29]. A number of studies have shown that NF- κ B activation protects cells from oxidative stress-induced damage [29-31]. In our study, RelA and RelB were activated in lung bronchial epithelial cells after exposure to

40% oxygen. This represents the activation of both the classical and non-classical NF- κ B pathways.

Additionally, a study by Al-Sadi et al. found that activation of NF- κ B induced by TNF- α can play a protective role [32]. Therefore, we speculated that moderate hyperoxia could activate the classic and non-classical pathways of NF- κ B. The TNF- α is an inflammatory cytokine that activates the NF- κ B pathway, and our results suggest that upregulated TNF- α activates the downstream NF- κ B pathway to play a protective role during moderate hyperoxia.

Mucosal immunity plays a key role in the innate immune system. Secretory immunoglobulin A (SIgA) is a first line of defense in the mucosal immune system. In mucus, SIgA plays an important role in neutralizing antigens and is generally present in polymers. Polymeric immunoglobulin A (pIgA) covalently binds to a polymeric immunoglobulin receptor (pIgR) through the J chain. The pIgR-pIgA complex is transcytosed across the cell. At the luminal surface, the pIgR is cleaved by a protease within a 42-amino acid region adjacent to the cell membrane, thus releasing SIgA into the lumen. pIgRs which do not bind to pIgA are secreted into the extracellular compartment and are referred to as Secretory Component (SC). Free SC is, therefore, present in extracellular fluid and blood [33]. The SIgA is also present in the lung and is sensitive to oxygen [34]. SC ensures selective transmembrane transport of IgA in the mucus and in the secretory glands of epithelial cells and SC is present in various endocrine fluids of the body and can neutralize endogenous pathogens [35]. The TNF- α can rapidly promote the secretion and activation of SC [9]. Expression of SC plays a major role in limiting the inflammatory response and promoting tissue repair [36]. In this study, SC protein expression after moderate hyperoxia was not significantly different compared with the control group, but SC mRNA levels were increased. Compared with the extreme hyperoxia group, SC protein and mRNA were significantly decreased in the moderate hyperoxia group. It is possible that SC secretion actually increased during moderate hyperoxia, but may have been consumed during the inflammatory reaction. This specific mechanism needs to be further studied.

Our results show that moderate hyperoxia can activate TNF- α and ASK1 in lung bronchial epithelial cells. It can also activate the NF- κ B pathway and promote the expression of SC, which plays a protective role. Not only were the lung bronchial epithelial cells undamaged by moderate hyperoxia, but cell proliferation occurred. These results provide theoretical support for the use of a moderate oxygen concentration as a clinical therapeutic.

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