

Original Article

Glucocorticoid attenuates hyperoxia-induced lung injury in neonatal rat and inhibits RAGE and NF- κ B expression

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Abstract: This study aimed to determine whether glucocorticoid therapy attenuates hyperoxia-induced lung injury and investigate the underlying mechanism. Twenty-four Sprague-Dawley newborn rats were randomly divided into three groups (n=8): sham control (control group); hyperoxia-induced acute lung injury (model group) and hyperoxia-induced acute lung injury treated with glucocorticoid (intervention group). Rats were sacrificed at 13 days^o after intervention. Receptor for advanced glycation end-products (RAGE) and nuclear factor- κ B (NF- κ B) expression in lung tissues was detected by PCR, Western blot and immunohistochemistry analysis. The levels of tumor necrosis factor α (TNF- α) and sRAGE in bronchoalveolar lavage fluid (BALF) and the serum were detected by enzyme-linked immunosorbent assay. The lung damage was evaluated by histological examination. Compared with model group, mRNA and protein levels of RAGE and NF- κ B in lung tissues were significantly lower in intervention group, while sRAGE and TNF- α levels in the serum and BALF were significantly lower in intervention group. In conclusion, RAGE/NF- κ B pathway plays an important role in hyperoxia-induced lung injury. Glucocorticoid may play a protective role against hyperoxia-induced lung injury via the inhibition of RAGE and NF- κ B expression.

Keywords: Glucocorticoid, receptor for advanced glycation end-products, lung injury, newborn

Introduction

Bronchopulmonary dysplasia (BPD) is one of the most common complications in premature children, and becomes one difficult problem in neonatal intensive care. Although the pathogenesis of BPD remains incompletely understood, neonatal lung injury caused by the exposure to oxygen toxicity in high oxygen environment is one of the important reasons [1]. High concentration of oxygen and oxidative stress play an important role in the occurrence and development of BPD [2, 3].

Recent studies have shown that type I alveolar epithelial cells are involved in the formation of pulmonary edema, alveolar and interstitial fibrin deposition, and affect the outcome and prognosis of acute lung injury and acute respiratory distress syndrome [4]. Receptor for advanced glycation end product (RAGE) is a multi-ligand receptor on cell surface and belongs to the immunoglobulin superfamily. RAGE has two forms: the first is full-length

RAGE which is usually located in type I alveolar epithelial cells (AECI); the second is soluble RAGE (sRAGE) which lacks a transmembrane domain and is released into the plasma and can be used as a potential injury marker of AECI. Indeed, increased level of circulating sRAGE is correlated to the severity of acute respiratory distress syndrome (ARDS) [5]. Our previous study showed that RAGE/NF- κ B pathway plays an important role in hyperoxia-induced lung injury [6].

Corticosteroids are widely used in neonatal BPD treatment. Corticosteroid reduces the dependence on oxygen, promotes extubation and shortens the duration of mechanical ventilation. However, there is still controversy on the use of corticosteroid because its mechanism of action remains unclear. This study aimed to determine whether glucocorticoid therapy attenuates hyperoxia-induced lung inflammation injury and investigate the underlying mechanism.

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Materials and methods

Animals

All animals were maintained in accordance with the guidelines of the NIH (Guide for the Care and Use of Laboratory Animals, 1996) and the animal experiments were performed under approved protocols of the Animal Care and Use Committee of Nanjing Medical University.

Newborn SD rats of clean grade (10-15 g) were provided by the Experimental Animal Center of Nanjing Medical University. The rats were randomly divided into hyperoxia model group, intervention group and control group (n=8). The rats in control group were exposed to normal air, while the rats in model group and intervention group were exposed to 95% oxygen concentration as described previously [7]. In addition, the rats in intervention group received intravenous injection of dexamethasone at 1 mg/(kg.d) every other day, and the rats in model group and control group were injected intravenously with the same volume of saline.

When the rats were 13 days old, they were weighed and received intraperitoneal injection of 10% chloral hydrate (8 ml/kg). All rats were sacrificed and blood samples were collected by eye-gouging method. In addition, bronchoalveolar lavage fluid (BALF) and lung tissues were collected for further analysis.

ELISA

TNF- α and sRAGE levels in serum and BALF were detected by using ELISA kits (Boster, Wuhan, China) following the manufacturer's instructions.

RT-PCR

Total mRNA was extracted from lung tissues using TRIzol reagent (Invitrogen, USA). cDNA was synthesized by using reverse transcription kit (TaKaRa, Dalian, China). The primers were synthesized by Shanghai Biological Engineering Company with the following sequences: RAGE 5' GGTGCTGGTTCTTGCTC 3' and 5' TCCCTCGCCTGTAGTT 3'; β -actin 5' GATGACAAGCAGCCTAT 3' and 5' TCCATGCCAATTTACAAC 3'; NF- κ B 5' GAAGAAGCGAGACCTGGAG 3' and 5' TCCGGAACACAATGGCCAC 3'. The relative mRNA levels of RAGE and NF- κ B were compared to that of β -actin GAPDH and calculated by $2^{-\Delta\Delta Ct}$

method. Each Ct value used for these calculations was the mean of the triplicate.

Western blot analysis

Lung homogenates were centrifuged at 10,000 rpm for 10 min, and the supernatants were collected for protein concentration measurement. Equal amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to cellulose acetate membranes. The membranes were blocked with 5% non-fat dry milk, and then incubated with antibodies for RAGE, NF- κ B and β -actin (Abcam, UK) at 4°C overnight. The membranes were washed and then incubated with secondary antibody (Cell Signaling, Danvers, MA, USA) for 1 h at room temperature. The bands were detected by using ECL kit (Pierce, Rockford, IL, USA) and exposed to X-ray film. The bands on X-ray films were quantified with image analysis system.

Pathological examination of lung tissues

Histological analysis of lung tissues was performed as described previously [8]. Lung injury evaluation was based on the scores of alveolar septum edema, alveolar hemorrhage, alveolar fibrin deposition, and alveolar cell infiltration. A five-point semiquantitative scoring system for lung injury severity was used as follows: negative=0, slight=1, moderate=2, high=3, and severe=4.

Statistical analysis

SPSS13.0 statistical software was used for statistical analysis, measurement data were represented as mean \pm SD. The differences among multiple groups were analyzed by One-way ANOVA analysis, comparison between two groups was analyzed by LSD method. $P < 0.05$ was considered statistically significant.

Results

General conditions of the rats

The activity of neonatal rats in model group and intervention group gradually decreased compared to control group, but all rats survived. The body weight of model group and intervention group was significantly lower than that of control group ($P < 0.05$), but the body weight between model group and intervention group

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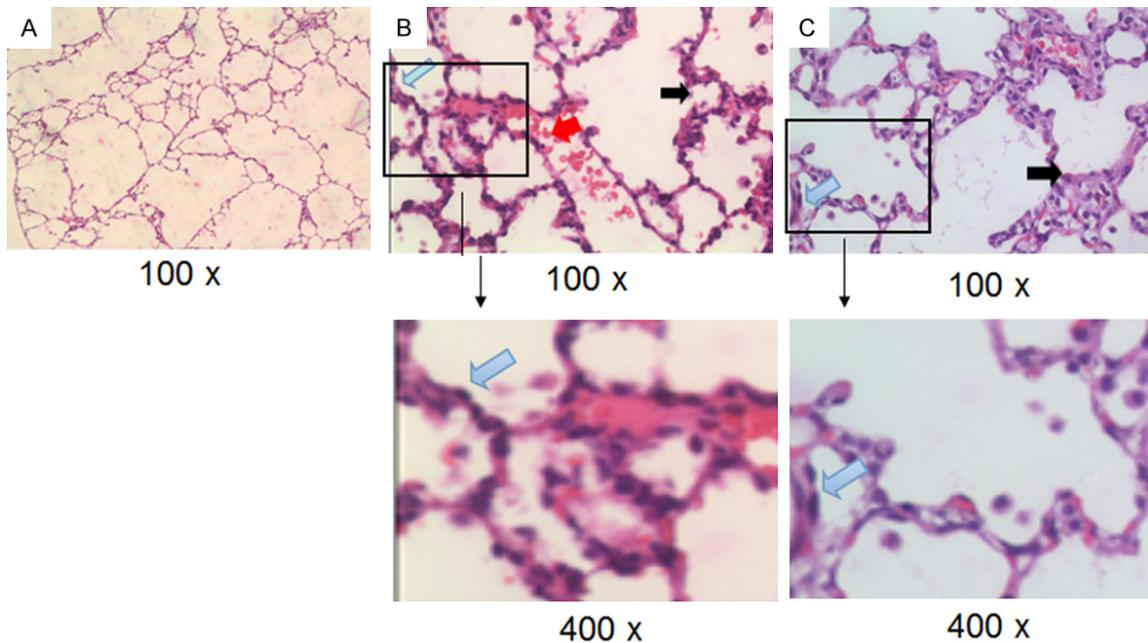


Figure 1. Pathological analysis of lung tissues of neonatal rats in each group. A: Control group, B: Hyperoxia model group, C: Intervention group. Arrows in blue indicated inflammatory cell infiltration, arrows in red indicated alveolar hemorrhage, arrows in black indicated alveolar wall edema and thickening. HE staining. Original magnification: $\times 100$ or $\times 400$.

Table 1. Lung injury score and RAGE and TNF- α levels in neonatal rats in each group (n=8)

Group	Lung injury score	TNF- α (ng/L)		sRAGE (ng/L)	
		Serum	BALF	Serum	BALF
Control	0.71 \pm 0.37	72.66 \pm 17.67	49.90 \pm 8.13	88.27 \pm 11.56	113.18 \pm 10.67
Intervention	1.06 \pm 0.37	127.50 \pm 9.70	76.73 \pm 10.68	145.78 \pm 14.46	109.73 \pm 15.26
Model	3.28 \pm 0.73	384.50 \pm 9.23	198.73 \pm 13.14	257.12 \pm 12.99	90.87 \pm 9.53
F value	37.68	1.35	428.27	345.85	7.91
P value	0.000	0.000	0.000	0.000	0.003

showed no significant difference ($P > 0.05$). These data indicate that intervention did not affect general condition of the rats.

Lung injury score

HE staining showed that the lung tissue had structural integrity and almost no exudation of inflammatory cells in control group. In contrast, inflammatory cell infiltration and bleeding, edema, alveolar septal thickening were observed in model group and intervention group, but these pathological changes were severe in model group than in intervention group (**Figure 1**). Lung injury score showed significant difference in three groups ($F = 37.68$, $P < 0.01$) (**Table 1**). These results demonstrated that intervention relieved lung injury.

TNF- α and sRAGE levels in serum and BALF

ELISA assay showed that serum levels of TNF- α and sRAGE were significantly different among the three groups ($F = 1.35$, $P < 0.01$; $F = 345.85$, $P < 0.01$). Serum levels of TNF- α and sRAGE were significantly higher in model group and intervention group than in control group, but were significantly lower in intervention group than in model group ($P < 0.05$) (**Table 1**). Similarly, ELISA assay showed that BALF levels of TNF- α and sRAGE were significantly different among the three groups ($F = 428.7$, $P < 0.01$; $F = 7.91$, $P < 0.01$). BALF levels of TNF- α and sRAGE were significantly higher in model group and intervention group than in control group, but were significantly lower in intervention group than in model group ($P < 0.05$) (**Table 1**).

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Table 2. RAGE and NF- κ B mRNA levels in lung tissues of neonatal rats in each group (n=8)

Group	RAGE mRNA	NF- κ B mRNA
Control	0.15 \pm 0.03	0.50 \pm 0.17
Model	0.39 \pm 0.09	0.90 \pm 0.36
Intervention	0.24 \pm 0.07	0.63 \pm 0.31
F value	24.84	3.85
P value	0.000	0.038

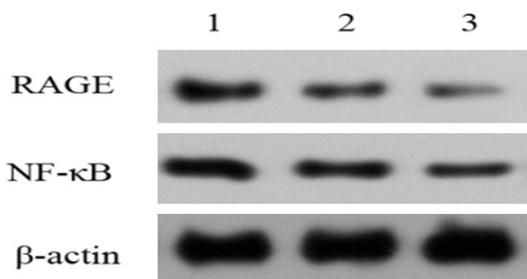


Figure 2. RAGE and NF- κ B protein expression in lung tissues of neonatal rats in each group. 1: Hyperoxia model group, 2: Intervention group, 3: Control group. Shown were representative blots from three independent experiments with similar results. β -actin was loading control.

These data indicate increased inflammation in model group but intervention could reduce inflammation caused by lung injury.

RAGE and NF- κ B expression in lung tissues

RT-PCR showed that mRNA levels of NF- κ B and RAGE in lung tissues were significantly different among the three groups (F=24.84, P<0.01; F=3.85, P<0.05); mRNA levels of NF- κ B and RAGE were significantly higher in model group and intervention group than in control group, but were significantly lower in intervention group than in model group (P<0.05) (**Table 2**).

Western blot analysis showed that protein levels of NF- κ B and RAGE were significantly higher in model group and intervention group than in control group, but were significantly lower in intervention group than in model group (**Figure 2**). These data indicate that intervention could reduce RAGE and NF- κ B expression.

Discussion

With the extensive application of mechanical ventilation and the advent of pulmonary surfactant, the survival rate of preterm children, especially children of low birth weight has been improved, but the incidence of BPD has increased recently. Studies suggest that the

pathogenesis of BPD is related to lung immaturity, high concentrations of oxygen, oxidative stress, infections, ventilator-associated lung injury and apoptosis [9, 10]. Systematic hormone therapy can improve lung function in the children, reduce the dependence on oxygen, promote extubation and shortens the duration of mechanical ventilation, and has been applied in the treatment of BPD. However, the use of corticosteroids may cause side effects, and long-term efficacy and mechanism of action remain controversial.

RAGE plays physiological role in alveolar gas exchange, cell spreading, cell proliferation, and extracellular matrix adhesion [11]. Under pathological conditions, the binding of the ligands to RAGE on the cell membrane would activate multiple signaling pathways, among which NF- κ B pathway is the most important [12]. The activation of RAGE/NF- κ B pathway and subsequent transcription of pro-inflammatory factors form a positive feedback loop to further increase RAGE expression, eventually leading to tissue damage [13]. Studies have shown that alveolar sRAGE level can be used as a marker for type I alveolar epithelial cell injury as well as lung injury [13]. Lizotte et al. found that RAGE content in lung tissue increased with the increase of lung maturity, while pathological lesions of BPD may be related to sRAGE or the imbalance of sRAGE/RAGE ratio [14].

Glucocorticoids can promote lung surfactant synthesis and lung antioxidant enzyme generation, reduce pulmonary edema and inflammation, reduce the infiltration of inflammatory cells and inhibit the proliferation of fibroblasts. Glucocorticoid has shown good efficacy for the treatment of BPD [15, 16], but the mechanism remains unclear.

In this study we found that after the application of glucocorticoid intervention, RAGE and NF- κ B expression at protein and mRNA levels were significantly decreased in hyperoxia exposed newborn rats. Thus we speculate that high oxygen stimulated the activation of RAGE/NF- κ B signaling pathway, causing lung damage. Glucocorticoid treatment could reduce the expression of pro-inflammatory factors and provide lung protection.

Previous studies have found that alveolar sRAGE level could be used as type I alveolar

epithelial cell injury marker [17]. This is consistent with the results of this study and support that sRAGE can be valued as hyperoxia-induced lung injury indicator. In addition, sRAGE serum level is closely related to chronic lung disease [18]. Therefore, we hypothesized that the level of sRAGE may be used as an index to monitor the progression and prognosis of BPD.

In summary, by inhibiting RAGE and NF- κ B expression glucocorticoid hormone may protect lung tissue from hyperoxia induced injury.

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Disclosure of conflict of interest

None.

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