Int J Clin Exp Med 2016;9(5):8387-8392
www.ijcem.com /ISSN:1940-5901/IJCEM0018165

Original Article

Effects of all-trans retinoic acid on expressions of receptor for advanced glycation endproducts and NF-κB in neonatal rats with hyperoxia-induced lung injury

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Received October 19, 2015; Accepted March 17, 2016; Epub May 15, 2016; Published May 30, 2016

Abstract: Background: The aim of this study was to explore changes in the receptor for advanced glycation endproducts (RAGE)-NF-κB signaling pathway of neonatal rats after hyperoxia exposure, and to evaluate the effects of intervention with all-trans retinoic acid (At-RA) on this pathway. Methods: Thirty neonatal rats were randomly divided into an air control group, a hyperoxia model group and an At-RA intervention group. The model group was exposed to a 95% oxygen atmosphere for one week immediately after birth, and the control group was exposed to the air in the same room. After the model was successfully established, the At-RA intervention group was intraperitoneally injected with At-RA (15 $mg \cdot kg^{-1} \cdot d^{-1}$) once every other day, three times in total. All rats were killed on the 13th day. The RAGE and NF-κB mRNA and protein expressions in lung tissues were detected with RT-PCR and Western blot respectively. Contents of TNF-α and soluble RAGE (sRAGE) in serum and bronchoalveolar lavage fluid (BALF) were detected with ELISA. Pathological changes of the lung tissues were evaluated with hematoxylin-eosin staining. Results: The model group had significantly higher RAGE and NF-κB mRNA and protein levels in lung tissues ($P < 0.05$) than those of control and At-RA intervention groups. This group also had significantly higher serum ($P < 0.01$) and significantly lower BALF ($P < 0.01$) sRAGE contents. Compared with the control group, the At-RA intervention group had significantly higher levels of RAGE and NF-κB mRNA and proteins ($P < 0.05$) in lung tissues, with significantly higher serum ($P < 0.05$) and significantly lower BALF ($P < 0.05$) sRAGE contents. Conclusion: The RAGE-NF-κB pathway, which was activated in neonatal rats with hyperoxia-induced lung injury, may be down-regulated by At-RA to exert a protective effect.

Keywords: All-trans retinoic acid, lung injury, RAGE-NF-κB pathway, neonatal rat

Introduction

Bronchopulmonary dysplasia (BPD), as the most common complication of premature infants, has mainly been attributed to lung injury induced by hyperoxia exposure [1]. Oxidative stress injury induced by high-concentration oxygen plays a critical role in the onset and development of BPD, which still lacks effective treatment strategies until now [2].

Type I alveolar epithelial cells participate in formation of pulmonary edema as well as depositions of alveolar and interstitial fibrin, and affect the development and prognosis of acute lung injury and acute respiratory distress syndrome [3]. Receptor for advanced glycation endproducts (RAGE), as a multi-ligand receptor, is a member of the immunoglobulin superfamily. Located in the basal cytoplasm of type I alveolar epithelial cells, RAGE marks their injury [4]. RAGE is classified into membrane and soluble types. The former is located in extramembrane, transmembrane and intramembrane domains, while the latter is released extracellularly into the cytoplasm, potentially marking the injury of type I alveolar epithelial cells. Increased sRAGE level in the circulatory system is correlated with the severity of acute respiratory distress syndrome [5]. Currently, the RAGE-NF-κB signaling pathway related to BPD pathogenesis has attracted particular attention due to the crucial role in hyperoxia-induced lung injury [6].

As the active metabolite of vitamin A, all-trans retinoic acid (At-RA) is mainly stored in lung tis-

sues and involved in lung development, maturation and repair [7]. At-RA can enhance the activity of alveolar epithelial cells in fetal rats in a hyperoxic environment, inhibit the cell apoptosis and improve the respiratory function [8], but the detailed mechanism remains elusive. In this study, we evaluated the effects of At-RA on the RAGE-NF-κB signaling pathway upon hyperoxia-induced lung injury, aiming to clarify the mechanism by which BPD can be treated.

Materials and methods

Reagents and apparatus

TRIzol kit, reverse transcription-polymerase chain reaction (RT-PCR) kit and ELISA kit were purchased from Life Technologies (USA). Anti-RAGE monoclonal antibody, anti-NF-κB antibody and anti-β-actin antibody were bought from Abcam (UK). CY-12C digital oxygen meter was obtained from Shanghai Longtuo Instrument and Equipment Co., Ltd. Primers were synthesized by Beijing BGI-GBI Biotech Co., Ltd.

Establishment of the neonatal rat model of hyperoxia-induced lung injury, and sample collection

Thirty 1-day-old SD rats of either gender weighing 10-15 g were provided by the Experimental Animal Center of our hospital. The rats were randomly divided into an air control group, a hyperoxia model group and an At-RA intervention group (n = 10). The model was established by exposing the rats in a 95% oxygen atmosphere for 7 d [9], and the control group was exposed to the air in the same room for 7 d. After modeling, the At-RA intervention group was intraperitoneally injected with At-RA (15 $mg \cdot kg^{-1} \cdot d^{-1}$) once every other day, three times in total. The other two groups were injected with normal saline with the same volumes. Mothers of the rats were awoken for breastfeeding every 24 h to prevent oxygen poisoning, and growth of the neonatal rats was observed.

All the rats were weighed on the 13th day and killed by intraperitoneal injection of 10% chloral hydrate (8 ml/kg). Blood (1-2 ml) was collected from eyeballs, and bloodletting was further performed by cutting off the abdominal aorta. The trachea in the middle of the neck was exposed, from which a small piece was scissored. Afterwards, a catheter was inserted and fixed. Then 1 mL of pre-cooled normal saline was slowly infused and sucked, and the procedure was repeated three times (recovery: 80%-90%). The bronchoalveolar lavage fluid (BALF) was collected. Subsequently, the thoracic cavity was rapidly scissored, from which bilateral lungs were taken out, observed by the naked eye and washed with normal saline. The left upper lobe was prepared into tissue homogenates, and the right upper lobe was fixed with 4% paraformaldehyde, paraffin-embedded, serially sectioned into 6 μm-thick, and subjected to immunohistochemical and histological examinations.

Detection of TNF-α and sRAGE contents in serum and BALF by ELISA

Blood was left still at room temperature for 2 h and then in a 4°C refrigerator for 3-4 h. After the blood coagulated and blood clot shrank, it was centrifuged at 4000 rpm for 10 min, from which the supernatant was collected and stored in a -20°C refrigerator. BALF was centrifuged at 4000 rpm for 10 min within 1 h, from which the supernatant was collected and stored in a -20°C refrigerator. TNF-α and sRAGE contents in serum and BALF were detected by ELISA according to the kit's instructions.

Detection of RAGE and NF-κB mRNA expression levels in lung tissue homogenates by RT-PCR

Total mRNA of lung tissues was extracted with the TRIzol method. RAGE primer sequences: Upstream: 5'-GGTGCTGGTTCTTGCTC-3', downstream: 5'-TCCCTCGCCTGTTAGTT-3', 235 bp in the length of amplification product. NF-κB primer sequences: Upstream: 5'-GAAGAAGCGAGACCTGGAG-3', downstream: 5'-TCCGGAACACAATGGCCAC-3', 398 bp in the length of amplification product. Primer sequences of internal reference β-actin: Upstream: 5'-GATGACAAGCAGCCCTAT-3', downstream: 5'-TCCATGCCAATTTACAAC-3', 450 bp in the length of amplification product. Three replicate wells were set for each sample. After the reaction, amplification and melting curves were recorded. Relative expression of mRNA = $2^{-\Delta\Delta CT}$. The mean value was used.

Detection of RAGE and NF-κB protein expression levels by Western blot

Lung tissue homogenates were centrifuged at 10000 rpm for 10 min, from which the super-

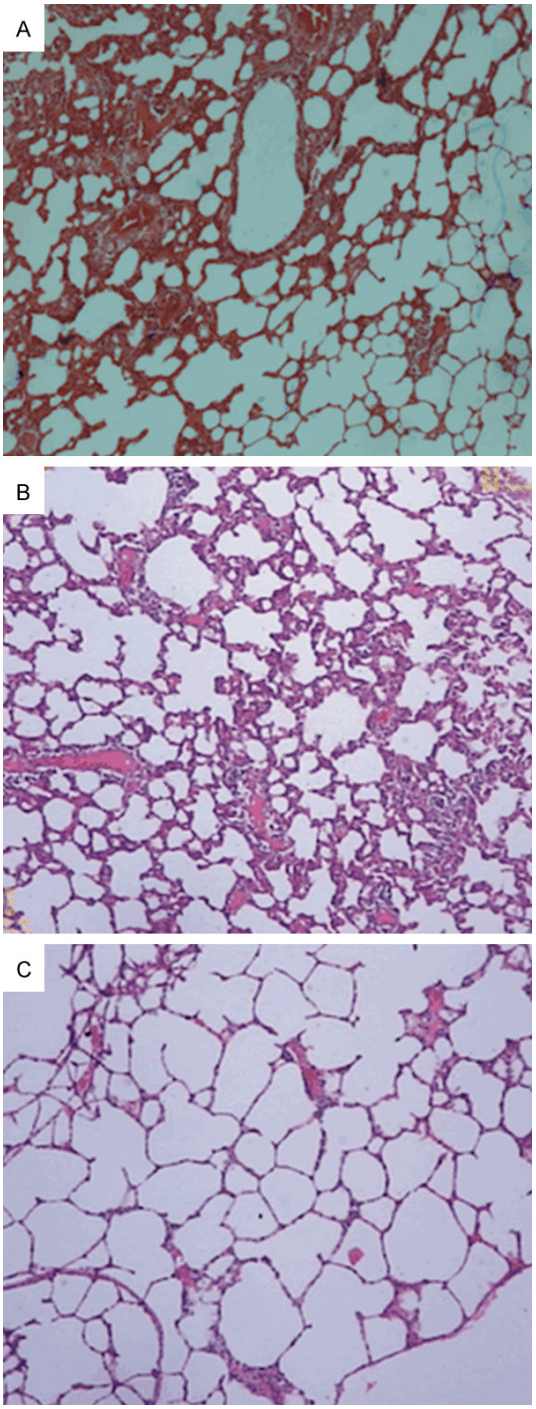

Figure 1. Pathological sections of lung tissues (hematoxylin-eosin staining, ×100). A. Control group; B. Model group; C. Intervention group. The control group had integral lung tissues, basically without exudation of inflammatory cells. However, there were exudation of inflammatory cells and thickening of alveolar septum edema in the model group, which were alleviated in the intervention group.

natant was collected to determine the protein concentration. Proteins were then quantified, loaded, fractioned with 10% SDS-PAGE and electronically transferred to a nitrocellulose membrane at 250 mA for 90 min. Subsequently, the membrane was blocked with 5% skimmed milk for 1 h, incubated overnight with RAGE primary antibody (1:1000) or NF-κB primary antibody (1:1200) at 4°C, washed three times with TBST (10 min each time), incubated with TBST-diluted, HRP-labeled secondary antibodies (1:4000) at room temperature for 1 h, washed three times again with TBST (5 min each time), and color-developed by ECL reagent. β-Actin was used as the internal reference.

Detection of pathological changes in lung tissues

Pathological changes of lung tissues were detected after hematoxylin-eosin staining. Under a light microscope, the pathological changes of lung injury were scored from 0 to 3 points according to edema of the alveolar septum, hemorrhage, fibrin deposition and cell infiltration. Four sections were selected for each rat, and ten non-overlapping visual fields were selected from each section. The mean of pathological scores of the four sections was used.

Statistical analysis

All data were analyzed by SPSS13.0. The categorical data were expressed as $\bar{x}\pm s$. Differences among groups were compared with one-way ANOVA. Pairwise comparisons were performed with LSD-t test. $P < 0.05$ was considered statistically significant.

Results

Growth of neonatal rats

The rats in model and intervention groups all survived, but they behaved sluggishly. Symptoms of the intervention group were relieved compared with those of the model group.

Scoring of lung injury

The control group had integral lung tissues, basically without exudation of inflammatory cells. In contrast, there were exudation of inflammatory cells and aggravated edema of the alveolar septum in the model group, which were alleviated in the At-RA intervention group

Table 1. TNF-α and sRAGE contents in serum and BALF

Group	Case No.	TNF-α (ng/L)		sRAGE (ng/L)	
		Serum	BALF	Serum	BALF
Control group	10	73.11±14.41	50.22±9.03	87.79±12.24	111.33±10.04
Model group	10	342±10.31[a]	202.17±13.34[a]	271.11±12.75[a]	90.39±10.92[a]
Intervention group	10	131.41±9.94[a,b]	77.74±11.39[a,b]	147.72±14.49[a,b]	112.24±14.55[a,b]
F value		1.41	433.12	344.73	7.76
P value		< 0.001	< 0.001	< 0.001	0.004

a: Compared with control group, P < 0.05; b: compared with model group, P < 0.05.

Table 2. Serum and BALF RAGE and NF-κB mRNA expressions ($\bar{x}$±s)

Group	Case No.	RAGE mRNA	NF-κB mRNA
Control group	10	0.17±0.07	0.52±0.12
Model group	10	0.42±0.11[a]	0.91±0.14[a]
Intervention group	10	0.27±0.04[a,b]	0.59±0.09[a,b]
F value		23.47	3.73
P value		< 0.001	0.034

a: Compared with control group, P < 0.05; b: compared with model group, P < 0.05.

(**Figure 1**). Lung injury scores of the three groups differed significantly (F = 36.77, P < 0.01), following a descending order of model group (3.44±0.57) > intervention group (1.12± 0.43) > control group (0.72±0.33) (P < 0.05).

TNF-α and sRAGE contents in serum and BALF

The serum TNF-α and sRAGE contents of the three groups were significantly different (P < 0.01), following a descending order of model group > intervention group > control group (P < 0.05). Meanwhile, the three groups also had significantly different TNF-α and sRAGE contents in BALF (P < 0.01). The TNF-α content of the intervention group significantly exceeded that of the control group, but the sRAGE content was lower. The intervention group had significantly lower TNF-α content and significantly higher sRAGE content than those of the model group (P < 0.05) (**Table 1**).

RAGE and NF-κB mRNA expressions in lung tissues

RAGE and NF-κB mRNA expressions in the lung tissues from the three groups differed significantly (P < 0.05), following a descending order of model group > intervention group > control group (P < 0.05) (**Table 2**).

RAGE and NF-κB protein expressions in lung tissues

RAGE and NF-κB protein expressions in the lung tissues from the three groups were significantly different (P < 0.01), also following a descending order of model group > intervention group > control group (P < 0.05) (**Figure 2**).

Discussion

Premature infants, especially those with extremely low birth weights, have enjoyed obviously increased survival rates owing to wide application of mechanical ventilation and clinical use of pulmonary surfactants. However, the incidence of BPD rises simultanesouly. BPD is mainly manifested as alveolar development disorders, being associated with lung immaturity, high oxygen concentration, infection, respirator-related lung injury and cell apoptosis [10].

RAGE is related with alveolar gas exchange, cell apoptosis and extracellular matrix adhesion [11]. In the pathological state, RAGE on the cell membrane can activate multiple signaling pathways after binding ligands. Particularly, the NF-κB pathway is most important. As a positive feedback loop, activation of the RAGE-NF-κB pathway and transcription of related proinflammatory cytokines increase the expression of RAGE gene on cell surface by positively regulating its promoter, thereby continuously activating and eventually injuring the cells [12]. The level of sRAGE, which indicates the injury of type I alveolar epithelial cells, can be used to assess the severity of lung injury and therapeutic effects [13]. RAGE content rises gradually along with the maturation of lung tissues, which, however, can be reduced by hyperoxia [14, 15], probably because the number of type

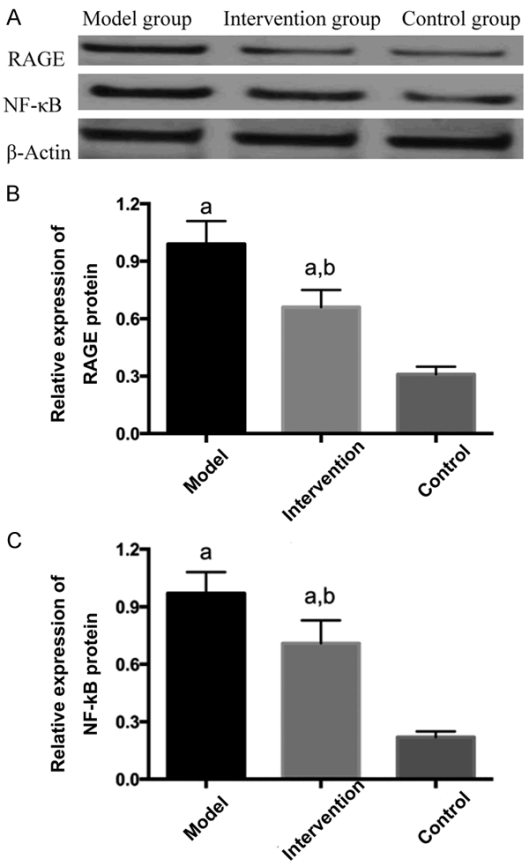

Figure 2. RAGE and NF-κB protein expressions in lung tissues. A. Electrophoretogram; B, C. relative protein expressions. a: Compared with control group, $P < 0.05$; b: compared with model group, $P < 0.05$.

I alveolar epithelial cells was decreased. Besides, the injury degree of BPD may be related with sRAGE loss or imbalance between sRAGE and RAGE.

At-RA, as the active metabolite of vitamin A, is mainly stored in lung tissues as the key signaling molecule of lung development, maturation and repair as well as pathophysiological processes of many diseases [7]. In the fetal period, maternal vitamin A enters fetal circulation through the placenta and is converted into At-RA by vitamin A enzyme in tracheal-bronchial cells. Afterwards, At-RA enters type I alveolar epithelial cells on the alveolar wall to regulate their growth, differentiation and development [16]. Controversially, At-RA may exert inhibitory [17, 18] or promotive effects on the proliferation of type I alveolar epithelial cells via insulin-like growth factor signaling [19]. Baybutt et al. [20] reported that the effects of At-RA on cell proliferation depended on the inoculation density, with high density being inhibitory and low density being promotive.

In this study, intervention with At-RA significantly decreased the expression levels RAGE, sRAGE and NF-κB mRNA and proteins in lung tissues, while significantly elevated serum sRAGE level. Hence, we postulated that hyperoxia initiated the RAGE-NF-κB signaling pathway and caused lung injury. At-RA treatment inhibited the expressions of RAGE and sRAGE and decreased those of proinflammatory cytokines, thus destroying the positive feedback loop and down-regulating this pathway to protect lungs. Being closely associated with lung tissues, sRAGE can block RAGE signal and prevent tissues and cells from ligand-related injury. Alveolar sRAGE level has been used as the injury marker of type I alveolar epithelial cells to evaluate the severity and clinical outcomes [21]. Moreover, sRAGE has also been closely related with chronic lung diseases in adults [22]. Accordingly, the severity and prognosis of BPD may be assessed by the level of sRAGE.

In summary, At-RA can protect lung tissues and feasibly treat BPD by down-regulating the RAGE-NF-κB pathway.

Disclosure of conflict of interest

None.

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