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Hyperoxia induces inflammation and regulates cytokine production in alveolar epithelium through TLR2/4-NF-κB-dependent mechanism

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Abstract. – OBJECTIVE: It has been reported that inflammation of lung could be induced by proinflammatory factor under hyperoxia, which may be attributed by increasing generation of reactive oxygen species (ROS).

MATERIALS AND METHODS: In the present study, with human epithelial lung cancer cell line A549 treated with hyperoxia as *in vitro* model, we found that hyperoxia stimulation induced TLR2/4 activity in A549 cells. ROS inhibitor NAC was used to investigate the role of ROS in hyperoxia-induced inflammatory cytokines secretion.

RESULTS: Results of mRNA to protein level showed that elevated TLR2/4 activity and hyperoxia-induced inflammatory cytokines secretion could be significantly attenuated by NAC. EMSA results showed the activation of nuclear factor-κB (NF-κB) increased after 2-h hyperoxia stimulation, and the ROS inhibitor blocked TLR2/4 and NF-κB activity.

CONCLUSIONS: Data suggested that the TLR2/4-NF-κB pathway is involved in hyperoxia-induced inflammatory cytokines secretion in A549 human type II alveolar epithelial cells.

Key Words:
Hyperoxia, Reactive oxygen species, Toll-like receptors, Inflammation.

Introduction

Hyperoxia-induced acute lung injury (HALI) is caused by prolonged exposure to hyperoxia with an extensive inflammatory response and destruction of the alveolar-capillary barrier[1-3]. The oxygen toxicity is mediated by the production and accumulation of excessive ROS, which not only triggers lung epithelial programmed cell death, but also evokes pulmonary cells to increase the secretion of chemoattractants and other proinflammatory cytokines, leading to leukocyte recruitment to the lung and pulmonary injury[4-7]. Although the mechanisms by which hyperoxia mediates the respond inflammation are not well defined, it is known that TLRs and NF-κB pathways are implicated in the inflammation induced by a variety of oxidant stresses.

The TLR family, as a class of pathogen-recognition receptors, provides critical first line defense against pulmonary infection through recognition of unique microbial structures and initiation of an inflammatory and adaptive immune response[8,9]. The essential role of TLRs in infectious pulmonary diseases with various microbes has been confirmed with mice model with single gene knockdown[10-12], However, little is known about the expression and function of TLRs in noninfectious pulmonary inflammation. Recent studies suggested that TLRs participate in response to oxidant stress, and ROS have been linked to TLR4 inflammatory signaling in neutrophils[13]. Moreover, mice with TLR2 and TLR4 gene deletion were more sensitive to hyperoxia and their survival time was half reduced compared with wild type[14]. TLR4-deficient mice are predisposed to lung injury and death following prolonged hyperoxia[15]. Activation TLR4 confers resistance to hyperoxia-induced pulmonary apoptosis with transgenic mice [16].

The present study was conducted to determine the signal transduction pathway involved in hyperoxia-induced lung epithelial cell respond inflammation. We examined the production of ROS, activation of TLRs and NF-κB pathways leading to the release of chemoattractants and other proinflammatory cytokines. Our results indicated that the elevated level of ROS resulting from hyperoxia exposure may act as the initiator of inflammation in lung epithelia cells. Increased level of ROS is involved in the activation of the TLRs- NF-κB signaling pathway, which leads to the release of IL-6 and IL-8 to execute the respond inflammation process.

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Materials and Methods

Cell Cultures

Human lung adenocarcinoma A549 cells (American Type Culture Collection CCL185) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NJ, USA)/F-12 supplemented with 10% fetal calf serum (FCS, Gibco BRL, Grand Island, NJ, USA), 100 units/mL of penicillin and 100 mg/mL of streptomycin. The cells were kept in a humidified 37°C incubator. Cells were grown to confluence in a six-well microplate and then cells were disaggregated in trypsin solution, washed with DMEM, centrifuged at 125 ×g for 5 min, then resuspended and subcultured according to standard protocols. Before being treated with hyperoxia, the cells were washed with DMEM/F-12 twice and maintained in DMEM/F-12 without FBS at 37°C. Cells were pretreated with a ROS scavenger (10 mM N-Acetyl L-Cysteine, NAC), if necessary, before the stimulation of hyperoxia for 6 h.

Hyperoxic Protocols

Hyperoxic exposures were performed by placing cells in sealed modular chambers (Billups-Rothenberg, Del Mar, CA, USA) and flushing the chambers with a gas mixture of 95% O_2-5% CO_2 at 1 l/min for 30 min. The entry and exit ports of the chambers were subsequently clamped, and the chambers were placed in a 37°C incubator for 2 h, 6 h, 12 h, 24 h and 48 h. In experiments involving hyperoxic exposures >24 h, the chambers were flushed with the 95% O2-5% CO_2 gas mixture every 24 h. pH of the cell culture medium was monitored to ensure that there was no significant CO_2 accumulation during the 24-h period between flushes.

RT-PCR and Real-Time RT-PCR

Total RNA samples were isolated from A549 cells using TRIzol reagent (Invitrogen, Shanghai, China). cDNA and RT-PCR samples were prepared according to the manufacturer's instructions (TOYOBO, Osaka, Japan). PCR amplification was performed with DNA polymerase for 38 cycles at 94°C for 1 min, at 60°C for 1 min, and at 72°C for 1 min using a commercial apparatus (GeneAmp; PerkinElmer Cetus, Waltham, MA, USA). The primers used in this study are listed in Figure 1. The product of PCR was assessed by electrophoresis in ethidium bromide-stained, 1.5% agarose gels. Real-time PCR analyses were performed with ABI Prism 7,700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using SYBR Green I PCR reagents (Applied Biosystems, Foster City, CA, USA) as previously reported[15,16]. To determine exact copy numbers of the target genes, quantified concentrations of subcloned PCR fragments of TLR2, and 4 were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 5 ng of total RNA samples were used for each real-time RT-PCR. Data were normalized with glyceraldehyde-3-phosphate dehydrogenase levels in each sample.

Measurement of Intracellular ROS

Cells were treated with hyperoxia, the medium was aspirated and the cells were washed twice with PBS and incubated with fresh culture medium without serum containing DCFH-DA, then diluted to a final concentration of 10 μM for 20 min at 37°C. The cells in six-well were collected and centrifuged, and then cell deposit was washed twice with ice-cold PBS. The fluorescent signal intensity of DCF was detected by flow cytometry (excitation at 488 nm and emission at 610 nm).

Flow Cytometric Analysis

A549 cells were treated with hyperoxia. Cell surface expression of TLR2 and TLR4 was examined by flow cytometry. Cells were incubated with the FITC-conjugated mouse anti-human TLR2 (TL2.1), TLR4 (HTA125) mAb (AbD Serotec, Oxford, UK), isotype control mouse IgG2a (BD Pharmingen, San Diego, CA, USA) for 1 h at room temperature. For intracellular FACS, the cell fixation/permeabilization kit (BD Pharmingen, San Diego, CA, USA) was used. Cells were washed once with PBS, and then incubated with the respective FITC-conjugated mAbs as described above for 1 h at room temperature. Stained cells were analyzed by a flow cytometer equipped with the manufacturer's software (CellQuest; Becton Dickinson, San Jose, CA, USA) for data acquisition and analysis.

Electrophoretic Mobility Shift Assay (EMSA)

A549 cells were cultured in a 10-cm dish in DMEM free FCS then stimulated with hyperoxia at indicated time intervals. The cells were collected on ice before isolation of nuclear extracts according to the protocol reported by Muller and Homaidan[17,18]. Briefly, cells (10^6/mL) were washed with ice-cold PBS, suspended in 200 μL of lysis buffer (10 mM HEPES [pH7.9], 10mM KCl, 0.1

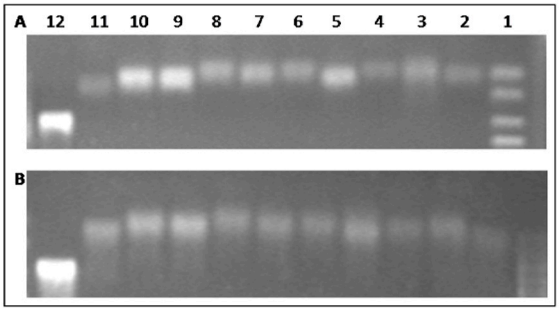

Figure 1. Expression of TLR family was examined by RT-PCR A: PBMC B: A549 cells. 1: DNA ladder 2: TLRl 3: TLR2 4: TLR3 5: TLR4 6: TLR5 7: TLR6 8: TLR7 9: TLR8 10: TLR9 11: TLR10 12: GAPDH.

mM EDTA, 0.1 mM EGTA, 1mM DTT) and allowed to swell on ice for 15 minutes, and then 12.5 µL of 10% Nonidet P-40 was added. The tube was then mixed thoroughly with a Vortex mixer for 10 seconds prior to centrifugation (20,000 g) at 4°C for 5 minutes. The nuclear pellets thus obtained were resuspended in 25 µL of ice-cold nuclear extraction buffer (20 mM HEPES [pH7.9], 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 m MDTT) and kept on ice for 15 minutes with intermittent agitation. The samples were subjected to centrifugation for 5 minutes at 4°C, and the supernatant was stored at −70°C after measurement of its protein content with the Bio-Rad protein assay kit (Bio-Rad, Munich, Germany). The detection of the activated NF-κB in the nuclei of unstimulated and stimulated cells was completed by using a biotin-labeled EMSA kit (Viagene, Ningbo, China), according to the manufacture's instruction. The consensus NF-κB oligonucleotides included in the kit was 5'-AGT TGAGGG GAC TTT CCC AGG C-3'.

IL-6 and IL-8 ELISA

Cells were exposed to the experimental conditions, and supernatants were harvested after treatment. Immunoreactive IL-6 and IL-8 levels were determined with a commercially available human IL-6 and IL-8 ELISA kit (Biosource International, Camarillo, CA, USA). All procedures were performed according to the manufacturer's instructions.

Statistical Analysis

All data were expressed as mean±SEM and assessed by ANOVA. Differences were pinpointed using Student-Newman-Keuls' test. All statistical analysis was conducted using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ and $p<0.01$ were considered to be statistically significant.

Results

mRNA Expression of the TLR Family in Unstimulated A549 cells

Spontaneous mRNA expression of the TLR family in unstimulated respiratory epithelial cells was tested by RT-PCR. As shown in Figure 1B, PBMC (Figure 1A) expressed all members of the TLR family (1-10) and A549 cells showed similar patterns of TLR family expression.

TLR2 and TLR4 Intracellularly Instead of on Cell Surface

TLR2 and TLR4 location was detected by flow cytometry with isotypic as a control antibody. Expressions of TLR-2 and TLR-4 on A549 cell surface were 1.20950±0.18597 and 3.5390±2.1040, respectively; and the intracellular expressions were 3.4671±0.905 and 11.05540±2.5850, respectively (Figures 2, 3). FACS analysis showed that TLR2 and TLR4 were mainly expressed intracellularly in A549 cells.

Hyperoxia-induced Intracellular ROS Generation

Intracellular ROS generation is known to be increased in cells under hyperoxic conditions[19, 20]. We measured the intracellular ROS generation in hyperoxia-treated A549 cells using DCFH-DA, and results showed that hyperoxia increased ROS generation in a time-dependent manner, with a significant increase of intracellular ROS detected after 6 h (Figures 4, 5).

TLR2 and TLR4 mRNA and Protein Expression Increased Under Hyperoxia

TLR2 and TLR4 expression increased at the mRNA and protein level with ROS generation under high concentration of hyperoxia (Figure 6), with a significant increase of protein expression detected at 6 h compared with the untreated control (Figure 7).

Hyperoxia-enhanced ROS-mediated NF-kB Nuclear Translocation

NF-κB is activated in hypoxic conditions and regulated by intracellular ROS (21,22). Results showed that hyperoxia increased nuclear translocation of NF-kB as demonstrated by the formation of an NF-kB/DNA complex within 12 h of treatment (Figure 8A). Co-incubation of nuclear extracts with an anti-NF-kB (p65) antibody caused a supershift of the NF-kB/DNA complex, confirming the specificity of binding (Figure 4A).

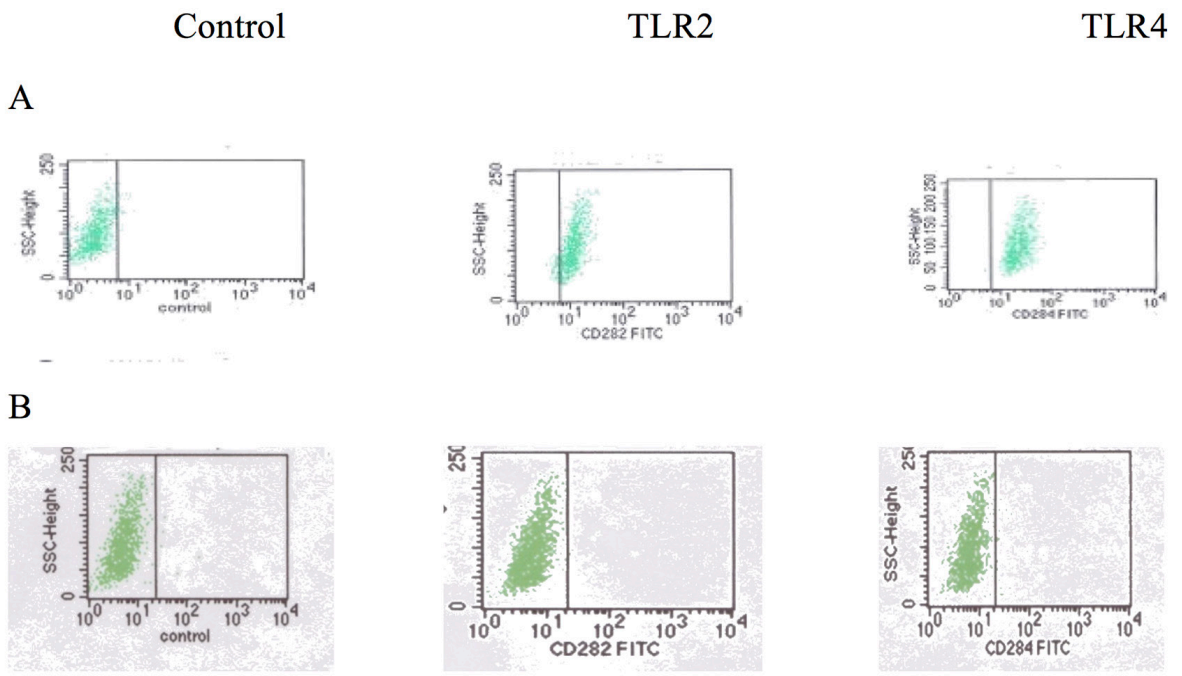

Figure 2. Expression and distribution of TLR2 and TLR4 protein in A549 cells. TLR2 and TLR4 are expressed intracellularly instead of on cell surface. ***A,*** intracellular ***B,*** cell surface.

Co-incubation of nuclear extracts with 100-fold molar excess of cold oligonucleotide inhibited the formation of the NF-kB/DNA complex after hyperoxia treatment, which further confirmed about the specificity of binding (Figure 8B). These data demonstrated that induction of the oxidative stress response by different time stimuli increases ROS-mediated NF-kB nuclear translocation in cultured A549 cells.

Hyperoxia Increased IL-6 and IL-8 Protein Levels

To confirm the functional expression of TLR2 and TLR4 on A549 cells, cells were treated with hyperoxia and the concentration of IL-6 and IL-8 in the culture supernatants was determined by enzyme-linked immunosorbent assay at 2, 6, 12, 24 and 48 h after treatment. As shown in Figure 9, the concentration of IL-6 and IL-8 protein was increased in a time-dependent manner by hyperoxia stimulation.

NAC Attenuated ROS-induced TLR2 and TLR4 Expression

ROS scavenger NAC (10 mM) significantly blocked hyperoxia-induced TLR2 and TLR4 expression (Figures 6, 7), indicating that ROS are separate pathways involved in hyperoxia-induced

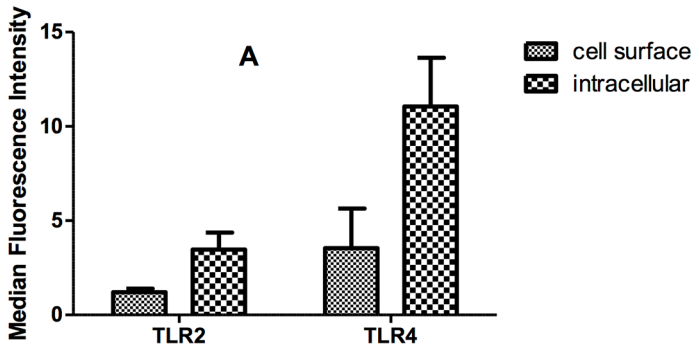

Figure 3. Expression and attribution of TLR2 and TLR4 protein in A549 cells.

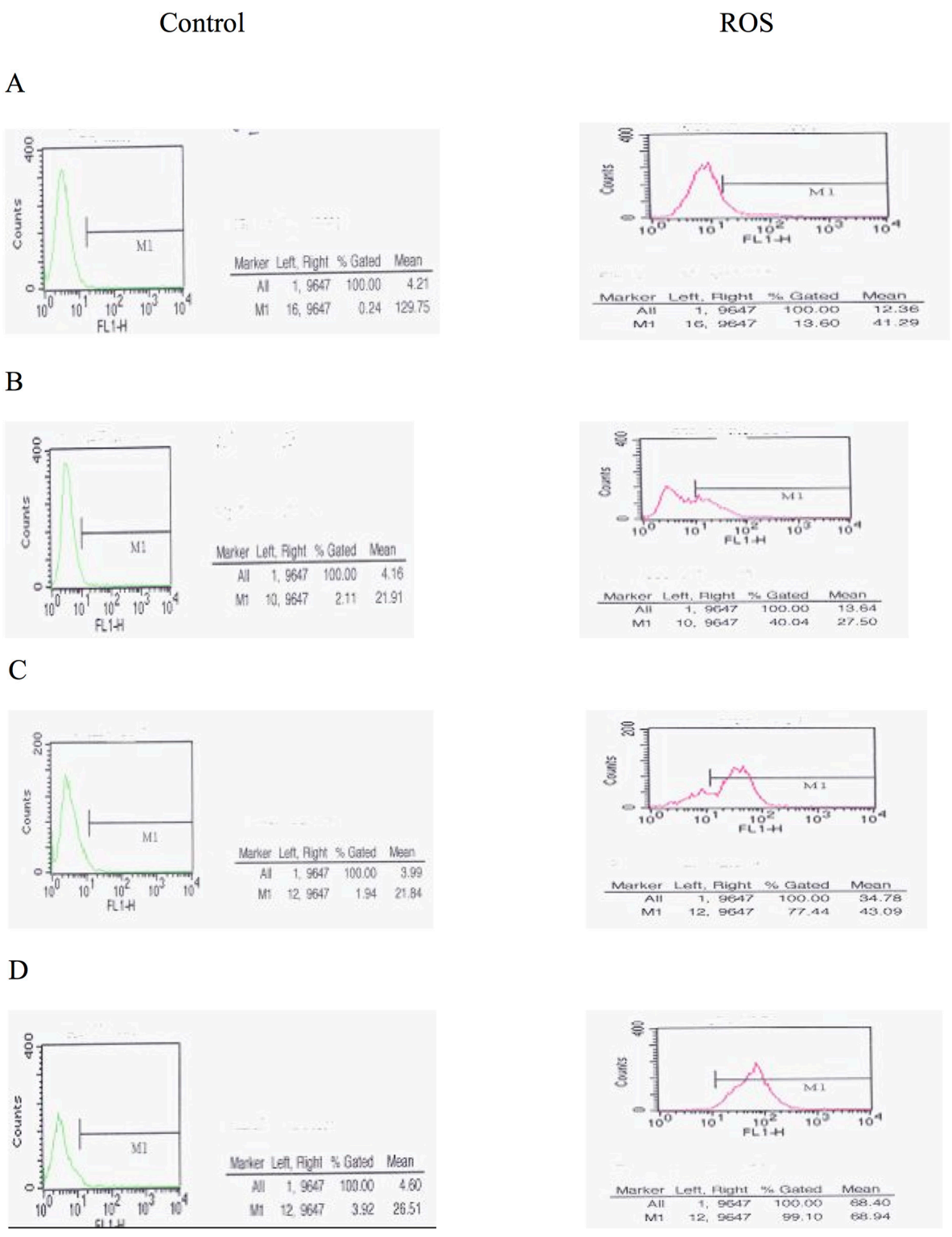

Figure 4. The effect on intracellular ROS of A549 exposed to hyperoxia and NAC.

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TLR2 and TLR4 expression. Enhanced expression of TLR2 and TLR4 induced by ROS is dependent on de novo transcription and translation. Hyperoxia-induced TLR2 and TLR4 mRNA level

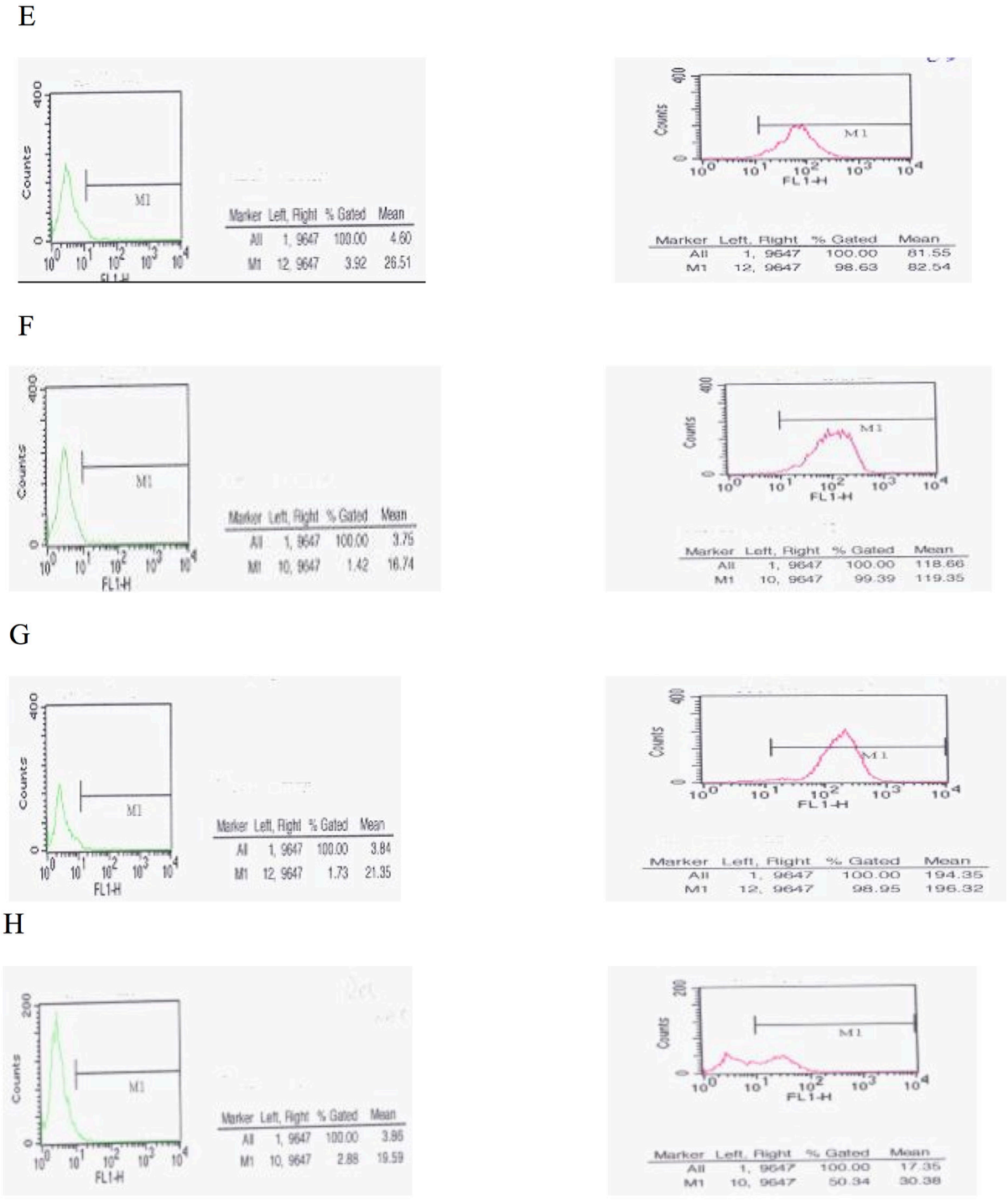

Figure 4. The effect on intracellular ROS of A549 exposed to hyperoxia and NAC.

was attenuated by NAC (Figure 6). Flow cytometry revealed that the intracellular TLR2 and TLR4 expression induced by hyperoxia was attenuated by pretreatment of NAC (Figure 7).

NAC Blocked TLR2- and TLR4-mediated Activation of NF-κB and IL-6/8

Cells were treated with NAC before being incubated with 6-h hyperoxia and NF-κB activity

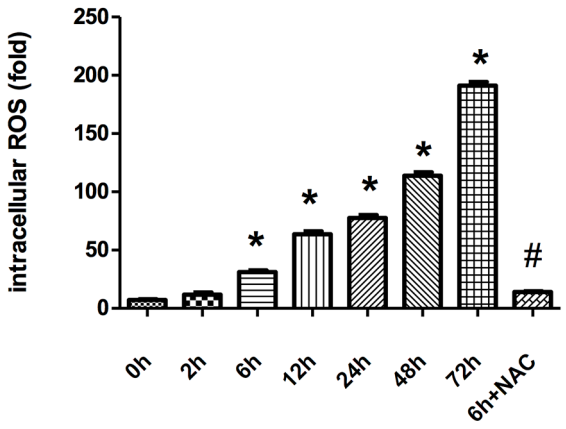

Figure 5. The effect on intracellular ROS of A549 exposed to hyperoxia and NAC ($^{*}p<0.05$ vs. untreated cells, $^{\#}p<0.05$ vs. hyperoxia for 6 h).

was measured. ROS scavenger greatly attenuated hyperoxia -induced NF-κB activity (Figure 8A). These results imply that ROS stimulation TLR-mediated NF-κB activation. Hyperoxia-induced activation of the IL-6 and IL-8 protein in A549 cells through the TLR2 and TLR4 was also significantly inhibited by NAC (Figure 9).

Discussion

Exposure to high oxygen concentration causes direct oxidative cell damage through increased production of reactive oxygen species. Hyperoxia-induced lung injury can be considered as a bimodal process resulting (1) from direct oxygen toxicity and (2) from the accumulation of inflammatory mediators within the lungs. While the in vitro response to oxygen seems to be cell type-dependent in tissue cultures, it is still unclear which are the death mechanisms and pathways implicated *in vivo*[23]. Although mounting evidence indicated that intracellular ROS is implicated in propagating the inflammatory response to hyperoxia stress of the innate immune system, there are no published data of its direct effects on parenchymal cells. We here showed for the first time that intracellular ROS, when treated to human alveolar epithelial cells, induced the inflammatory cascade by increasing IL-6 and IL-8 protein expression via activation of the NF-κB pathway.

One interesting concept that has arisen from the investigations into intracellular ROS is the idea of ROS as an endogenous TLR2/4 ligand[13,24]. Considering that the TLR2/4 and NF-κB pathways are shared by LPS and ROS, similar pathophysiologic responses may be induced in infectious and non-infectious mechanisms of ALI. Any stress to the lung, such as ischemia, heat and hypoxia, could lead to excessive product of intracellular ROS, which subsequently activate the TLR4 receptor pathway and modulate the inflammatory cascade coherently with infection and LPS stimulation.

The background information may help explain the similar responses occurred in LPS-induced lung injury and other forms of ALI, yet debate and controversy results were reported about the role of oxidants in activation of NF-κB. In the present study, we found that the hyperoxia-in-

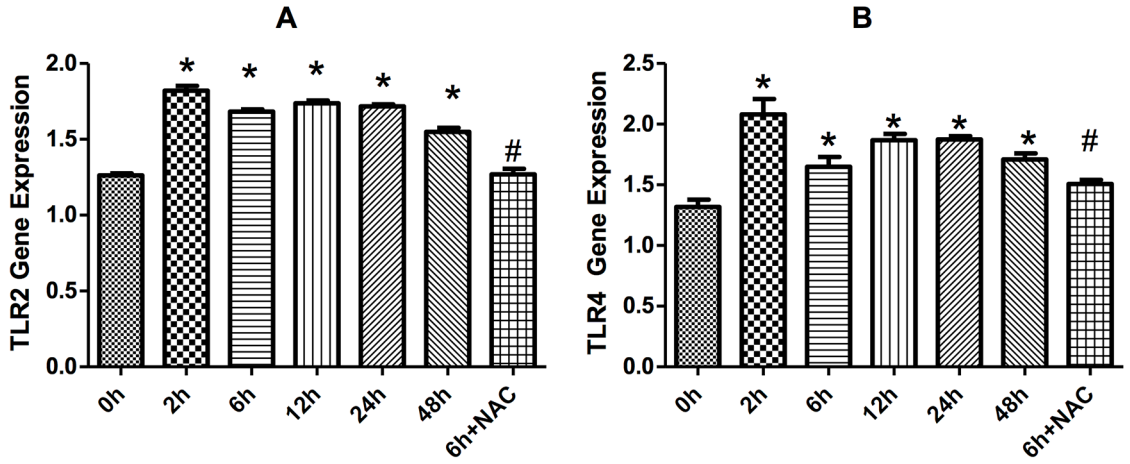

Figure 6. The effect on TLR2 and TLR4 mRNA expression of A549 exposed to hyperoxia and NAC ($^{*}p<0.05$ vs. untreated cells, $^{\#}p<0.05$ vs. hyperoxia for 6 h).

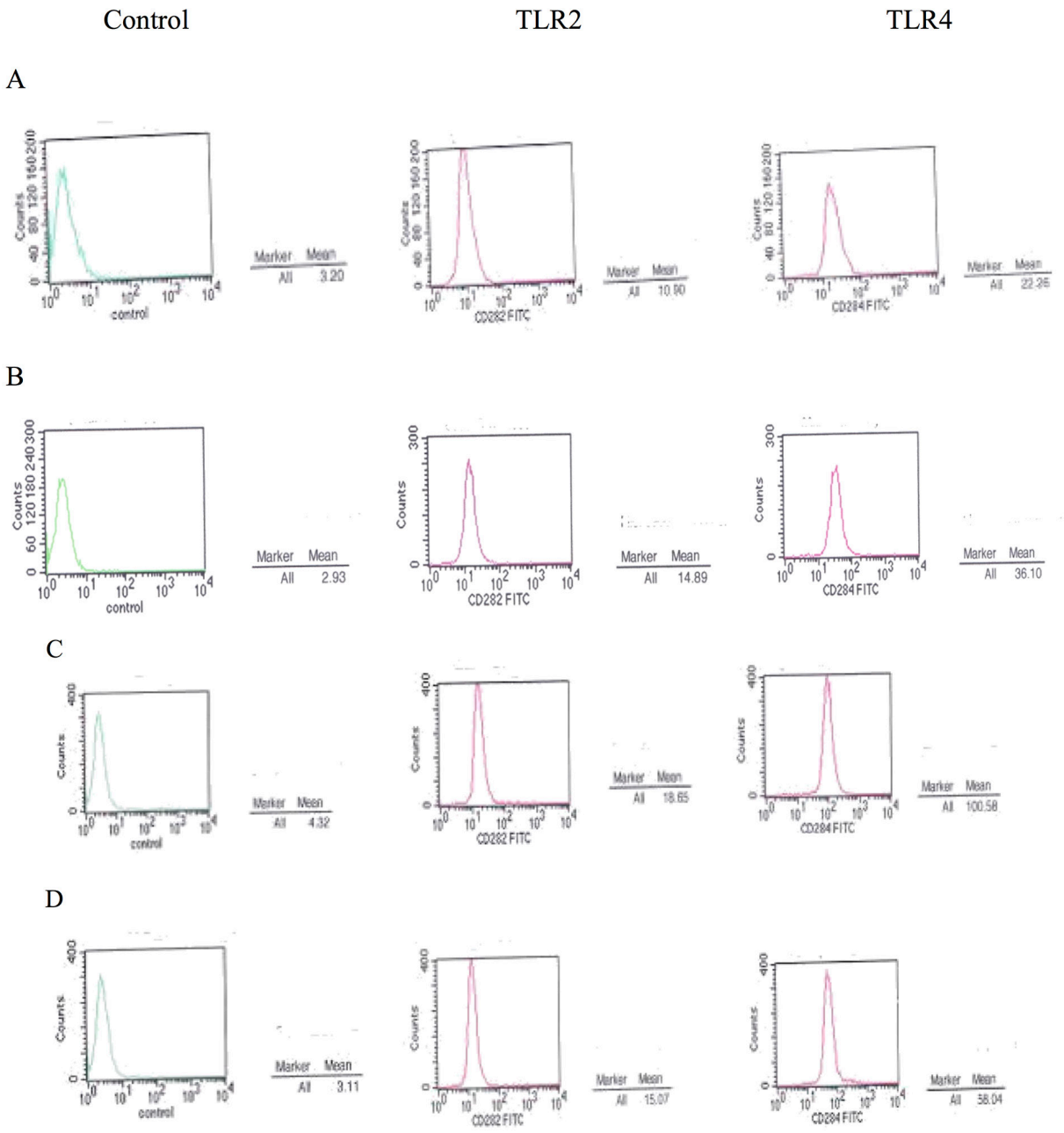

Figure 7. The effect on TLR2 and TLR4 protein expression of A549 exposed to hyperoxia and NAC. ***A,*** Air group; ***B,*** hyperoxia for 2hrs group; ***C,*** hyperoxia for 6hrs group; ***D,*** hyperoxia for 12hrs group.

Continued

duced oxidative stress response increases NF-κB nuclear translocation in cultured A549 cells. The oxidative stress involved in the activation of NF-κB has also been indicated by previous studies[25-27]. Evidence linking ROS to NF-κB based on complementary observations also demonstrated that nuclear translocation of NF-κB can be enhanced by exposure to ROS, and cytokine-induced activation of NF-κB can be prevented by antioxidants[25,26]. Although ROS can activate signaling pathways leading to NF-κB activation[28], the specific intracellular events affected by oxidant stress have not been completely defined yet.

As the mainly activation NF-κB signals, TLR2 and TLR4 recruits intracellular adaptor molecule MyD88 or engages an alternative Toll/IL-

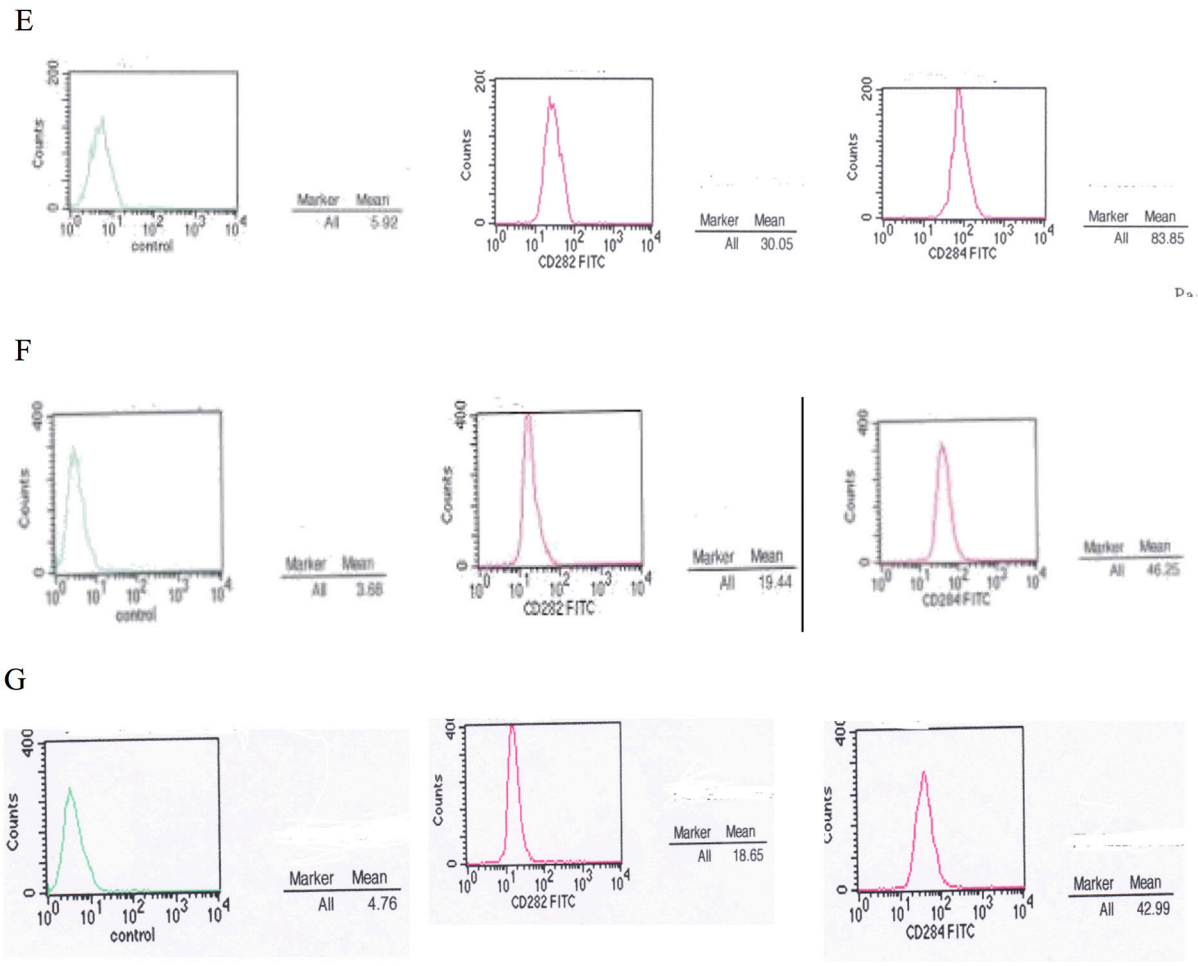

Figure 7. *(Continued).* ***E,*** hyperoxia for 24hrs group; ***F,*** hyperoxia for 48hrs group; ***G,*** hyperoxia for 6hrs and NAC group.

Table I. The sequence of primers used for RT-PCR.

cDNA	Forward	Reverse	bp
TLR1	CAGTGTCTGGTACACGCATGGT	TTTCAAAAACCGTGTCTGTTAAGAGA	105
TLR2	GGCCAGCAAATTACCTGTGTG	AGGCGGACATCCTGAACCT	67
TLR3	CCTGGTTTGTTAATTGGATTAACGA TLR4	TGAGGTGGAGTGTTGCAAAGG	82
TLR4	AATCTAGAGCACTTGGACCTTTCC	GGGTTCAGGGACAGGTCTAAAGA	115
TLR5	TGCCTTGAAGCCTTCAGTTATG	CCAACCACCACCATGATGAG	77
TLR6	GAAGAAGAACAACCCTTTAGGATAGC	AGGCAAACAAAATGGAAGCTT	88
TLR7	TTACCTGGATGGAAACCAGCTAC	TCAAGGCTGAGAAGCTGTAAGCTAG	72
TLR8	AGCGGATCTGTAAGAGCTCCATC	GCGGTAGCTCCGTGAATGAGTG	107
TLR9	GCAGTCAATGGCTCCCAGTTC	CCGTGAATCATTTTCAGTCAAGAC	106
TLR10	TTATGACAGCAGAGGGTGATGC	CTGGAGTTGAAAAAGGAGGTTATAGG	152
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATITC	226

1R domain-containing adaptor inducing IFN-β or TRIF. MyD88-dependent gene expression is mediated primarily by early phase activation of the transcription factor NF-κB and includes proinflammatory cytokines such as IL-6 and IL-8, whereas TRIF uses IFN regulatory factor 3 to

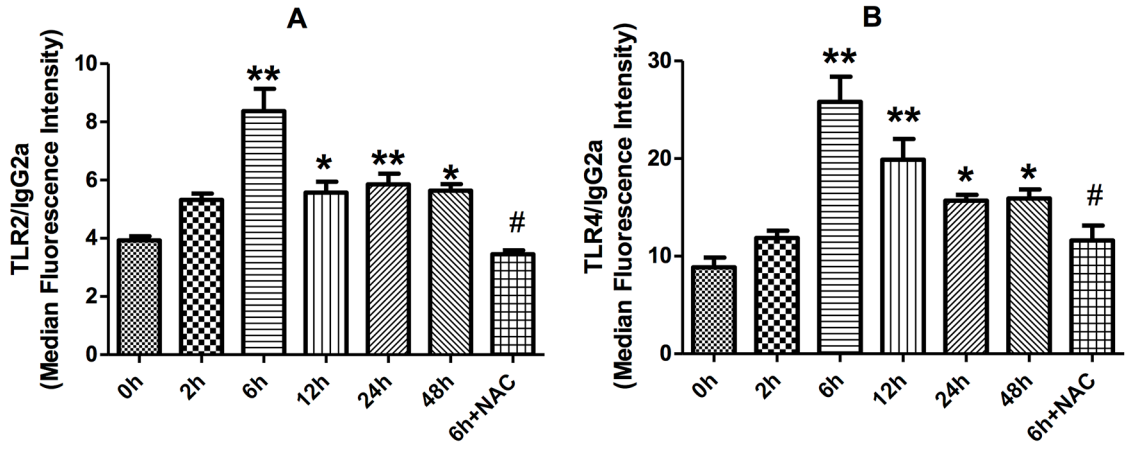

Figure 8. The effect on TLR2 and TLR4 protein expression of A549 exposed to hyperoxia and NAC. The time course for ROS-mediated nuclear accumulation of NF-kB in A549 cells is shown in Figure 8A. Hyperoxia increased nuclear translocation of NF-kB as demonstrated by formation of an NF-kB/DNA complex within 12 h of treatment (Figure 8A). Control cells did not have detectable NF-kB/DNA complexes (Figure 8A, lanes 1). Co-incubation of nuclear extracts with 100-fold molar excess of cold oligonucleotide inhibited formation of the NF-kB/DNA complex after hyperoxia treatment, which further confirmed about the specificity of binding (Figure 8B). ($^{*}p<0.05$, $^{**}p<0.01$ vs. untreated cells, $^{\#}p<0.05$ vs. hyperoxia for 6 h).

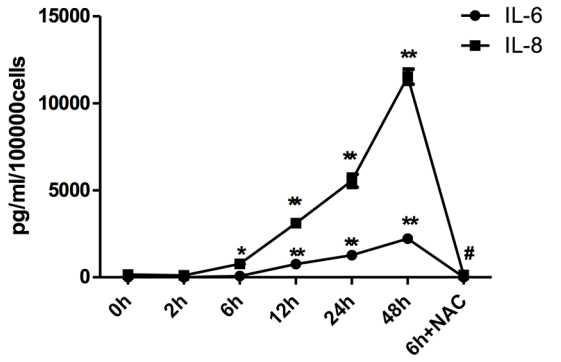

Figure 9. The effect on IL-6 and IL-8 protein level of A549 exposed to hyperoxia and NAC. ($^{*}p<0.05$, $^{**}p<0.01$ vs. untreated cells, $^{\#}p<0.05$ vs. hyperoxia for 6 h).

induce IFN-β and IFN-dependent gene products and contributes to the late phase of NF-κB activation[29].

The location of TLR2 and TLR4 mRNA and protein in airway epithelial cells are reported with discordant data. Armstrong et al[30] and Gon et al[31] reported cell surface expression of TLR2 and TLR4; while others[32] observed the absence of TLR4 associated with endotoxin hyporesponsiveness of intestinal epithelial cells, which is not directly involved in inflammation of the intestinal epithelium. A more recent study by Guillot et al[33] demonstrated the intracellular distribution of TLR2 and TLR4 on pulmonary epithelial cells by means of complementary techniques including flow cytometry, biotinylation/precipitation, and confocal microscopy using both cell lines and primary cells. In the present study, we investigated the expression of the TLR family in respiratory epithelial cells measured by RT-PCR. FACS analyses showed that human alveolar epithelial cells express TLR2 and TLR4 mainly intracellular, but not on the cell surface, even under stimulation with high concentration oxygen. These findings suggested that it is possible for airway surface epithelial cell-associated mucosal immune system to create an immunosilent condition for TLR-mediated innate immunity to prevent unnecessary inflammatory responses to normal bacterial flora.

We focused on the change of ROS within the intracellular environment and its effect on proteins of TLR family as well as proinflammatory factors, in which we found that intracellular ROS significantly increased in A549 cells treated with prolong hyperoxia. When investigating the up-regulated TLR2 and TLR4 mRNA and protein expression, proinflammatory cytokines IL-6 and IL-8, the two important products after activation of TLR2 and TLR4, increased significantly after hyperoxia. Our data are supported by a previous study by Imai et al[34]: Loss of TLR4 or TRIF expression protects mice from H5N1-induced ALI, and deletion of neutrophil cytosolic factor1 (NCF1), which controls ROS production, improves the severity of H5N1-mediated ALI.

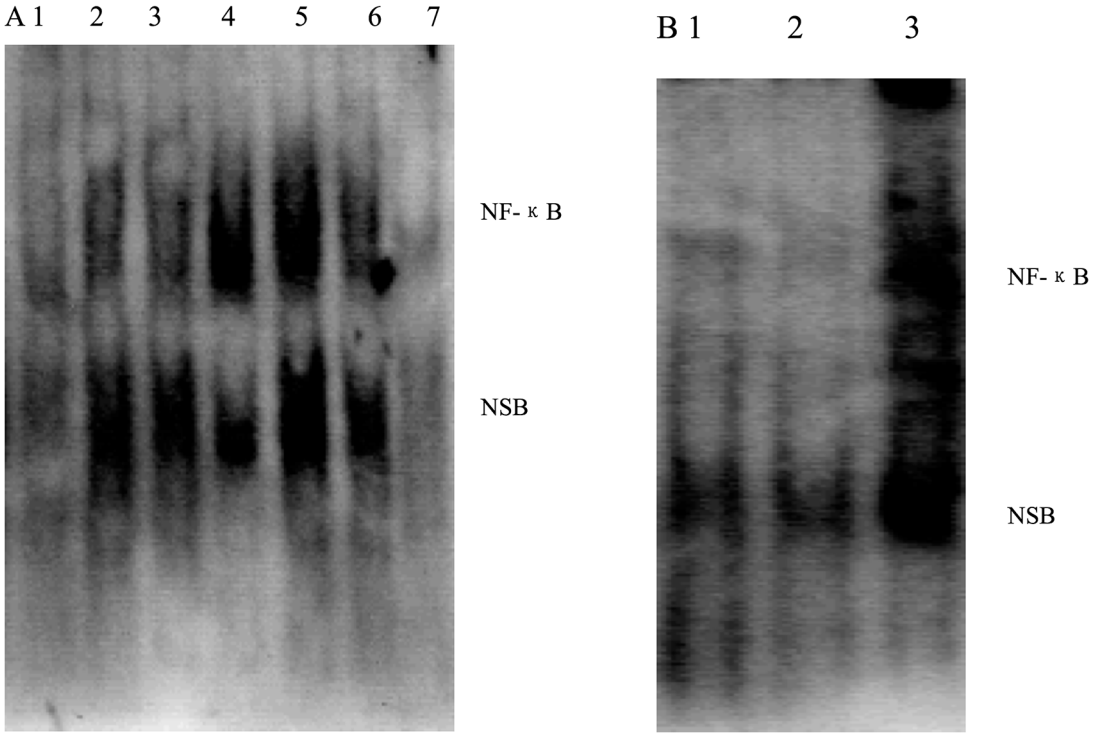

Figure 10. The effect on NF-κB activation of A549 exposed to hyperoxia and NAC: ***A,*** The result of an electrophoretic mobility shift assay (EMSA) obtained from nuclear extracts. From line 1 to 7: Air group, hyperoxia for 6 h and NAC group, hyperoxia for 2 h group, hyperoxia for 4h group, hyperoxia for 6 h group, hyperoxia for 8 h group, hyperoxia for 12 h group; ***B,*** Competitive inhibition test of NF-κB. From line 1 to 3: Air group, hyperoxia for 6h group, hyperoxia for 6 h and nonspecific for NF-kB competitor oligonucleotide probe group.

We also resulted that ROS scavenger attenuated TLR2- and TLR4-mediated activation of the NF-κB promoter, as well as NF-κB–dependent activation of the IL-6 and IL-8 promoter in A549 cells. These results indicated that ROS functions as a positive regulator of airway inflammation by counteracting the activation of TLR2 and TLR4 under hyperoxia.

Conclusions

The implications of our study are to provide further information for understanding mechanism involved in TLR induced synthesis of IL-8, which may bring new insight into the molecular pathogenesis of inflammatory hyperoxia-induced lung injury. Besides, our findings implied that ROS scavenger may function as a negative regulator of TLR signaling and may regulate innate immune homeostasis in the airway. Hopefully, the results may lead to the development of potential therapeutic strategies for inflammatory of hyperoxia-induced lung injury.

Conflicts of interest

The authors declare no conflicts of interest.

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