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Nrf2 regulates the expression of NOX1 in TNF-α-induced A549 cells

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Abstract
Acute lung injury causes severe inflammation and oxidative stress in lung tissues. In this study, we analyzed the potential regulatory role of nuclear factor erythroid-2-related factor 2 (Nrf2) on NADPH oxidase 1 (NOX1) in tumor necrosis factor-α (TNF-α)-induced inflammation and oxidative stress in human type II alveolar epithelial cells. In this study, A549 cells were transfected with Nrf2 siRNA and overexpression vectors for 6 h before being induced by TNF-α for 24 h. TNF-α upregulated the expression of NOX1 and Nrf2 in A549 cells. Furthermore, overexpression of Nrf2 could reduce TNF-α-induced NF-κB mRNA and protein expression after transfection with the Nrf2 siRNA vector, and the levels of IL-6, IL-8, ROS, and malondialdehyde (MDA) in TNF-α-induced A549 cells increased, while the level of total antioxidation capability (T-AOC) decreased. On the other hand, the overexpression of Nrf2 decreased the levels of IL-6, IL-8, ROS, and MDA, while increasing T-AOC. The mRNA and protein levels of NOX1 were dramatically increased by TNF-α, while those changes were notably suppressed by Nrf2 overexpression. Further studies demonstrated that Nrf2 suppressed NOX1 transcription by binding to the -1199 to -1189 bp (ATTACACAGCA) region of the NOX1 promoter in TNF-α-stimulated A549 cells. Our study suggests that Nrf2 may bind to and regulate NOX1 expression to antagonize TNF-α-induced inflammatory reaction and oxidative stress in A549 cells.
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Introduction

Acute lung injury (ALI) is a common and lethal complication of pulmonary or extrapulmonary diseases and accounts for an overall mortality rate of approximately 40%.[1,2] Moreover, ALI is the primary cause of death for numerous illnesses and has recently been shown to be the main cause of COVID-19-related mortality.[3,4] However, data on the pathogenesis of ALI remains scant. ALI presents diffused alveolar damage as the predominant pathological feature.[5] In type II alveolar epithelial cells (A549 cells), hyperactive inflammatory reaction and excessive oxidative stress are the major mechanisms perturbed by the ALI.[6] Thus, maintenance of the appropriate inflammatory response and oxidative stress in A549 cells is crucial in ALI therapy.

Imbalanced oxidative stress is mainly a function of dysregulated generation of reactive oxygen species (ROS) and excessive consumption of antioxidants.[7] Members of the nicotinamide adenine dinucleotide phosphate oxidase (NOX) family are the major contributors in the production of ROS, and NOX1 has been shown to maintain the production of ROS in the lungs. Besides, aberrant NOX1 expression in A549 cells results in excessive oxidative stress, thus contributing to the pathogenesis and development of ALI.[8] Therefore, reduction of the NOX1-mediated ROS production might effectively mediate the alleviation of inflammation and oxidative stress in the A549 cells under ALI. Nuclear factor erythroid-2-related factor 2 (Nrf2) is an important transcription factor that plays a crucial role in regulating the transcription of a wide range of antioxidant genes.[9,10] Under oxidative stress, the Nrf2-Keap1 complex is dissociated, which allows Nrf2 to translocate into the nucleus, resulting in the activation of its downstream genes, thus exerting its role in modulating the levels of oxidative stress, inflammation, apoptosis, and autophagy.[9] Previous studies have demonstrated the roles of activated Nrf2 in preventing the occurrence or mitigation of the severity of ALI or acute respiratory distress syndrome, and in the protection against ALI.[11–14]

Tumor necrosis factor (TNF)-α is not only a pivotal proinflammatory cytokine but also plays a crucial role in the triggering of inflammatory reactions and oxidative stress.[15,16] Hence, TNF-α has been widely used to establish ALI *in vitro* models.[17] It has been reported that NOX1 is critical for ROS production and leads to the activation of Nrf2 in A549 cells under conditions of intermittent hypoxia.[18] However, little is known about whether Nrf2 regulation affects NOX1 in ALI. In this study, we hypothesize that Nrf2 might also contribute a crucial role in modulating NOX1, involved in the pathogenesis and progression of ALI. This understanding would make the basis for the improvement of prognosis of ALI and validate potential therapeutic targets for the disorder.

Materials and Methods

Cell culture and treatment

A549 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum and incubated in an environment containing 5% CO_2 at 37°C. The cells were divided into three categories. Cells in the first category were divided into the control group and TNF-α-treated group; those in the second category were randomly divided into the naive group, TNF-α-treated group, negative control group, Nrf2 siRNA group, Nrf2 overexpression group, or empty vector group; while those in the third category were randomly divided into pGL3 group, pGL3-NOX1-1500 group, or pGL3-NOX1-1489 group.

Cells in the control group were cultured in a normal medium, while those in the TNF-α-treated group were cultured with 2.5, 5, 10, 20, or 40 ng/mL TNF-α for 24 h; or cultured with 10 ng/mL TNF-α for 0, 6, 12, 24, 36, or 48 h. Cells in the naive group were cultured in a normal medium, while those in the TNF-α-treated group were stimulated by 10 ng/mL TNF-α for 24 h. Vector transfection was performed using lipofectamine TM 2000. Cells in the remaining groups were transfected with negative siRNA vector (50 nmol/L), Nrf2 siRNA vector (50 nmol/L), Nrf2 high expression vector (4 μg), or empty vector (4 μg) for 6 h, followed by stimulation with 10 ng/mL TNF-α for 24 h. Cells in the third category were transfected with pGL3 basic plasmid, pGL3-NOX1-1500 plasmid, or pGL3-NOX1-1489 plasmid.

ELISA assay

Cell supernatants in each group were collected, and the concentration of IL-6 or IL-8 was estimated using ELISA, following the manufacturer's protocol. Each sample was analyzed in duplicates.

Detection of ROS

Intracellular ROS was determined by nonfluorescent probe 20, 70-dichlorofluorescein diacetate (DCFH-DA) by the manufacturer's protocol. Briefly, cells were digested with 0.25% trypsin-EDTA, followed by treatment with DCFH-DA (10 mM) in the dark at 37°C for 30 min. The cells were washed in phosphate-buffered saline (PBS) and then observed under a fluorescence microscope. Cellular fluorescence intensity was determined in a microplate reader (RT-7300, Rayto, Guangdong, China) at an excitation of 485 nm and an emission of 538 nm.

Evaluation of MDA and total antioxidation capability (T-AOC)

Total cellular protein was extracted by cell lysis buffer, supplemented with protease inhibitors, and then quantified using the bicinchoninic acid (BCA) assay, following the manufacturer's instructions. Afterward, MDA concentration was estimated using an MDA kit according to the manufacturer's protocol. On the other hand, the T-AOC was determined using the Rapid ABTS method as prescribed by the T-AOC assay kit. The mixtures were then measured at 405 nm by a Varioskan Flash spectral scanning multimode reader. The T-AOC level was expressed as umol/mg.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from the cell pellets using Trizol reagent solution following the manufacturer's instructions. The RNA concentration and purity were evaluated with a spectrophotometer. Thereafter, 600 ng of the total RNA was used to perform cDNA synthesis by reverse transcription PrimeScript™ RT reagent kit. The genes of interest were then amplified using an SYBRR premix Ex TaqTM reagent kit, following the manufacturer's instructions, using an ABI PRISM 7500 sequence detection system (Applied Biosystems, USA). The relative expression of target genes was normalized to that of GAPDH, a housekeeping gene, and calculated by the $2^{-\Delta\Delta Ct}$ method. The PCR primer sequences used were as below:

GAPDH forward, 5′-TGCCACTCCTCCACCTTTG3-′;
GAPDH reverse, 5′-CGAACCACCCTGTTGCTGT-′;
NF-κB forward, 5′-ACAACCCCTTCCAAGTTCCT-′;
NF-κB reverse, 5′-TGGTCCCGTGAAATACACCT-′;
Nrf2 forward, 5′-AAACCAGTGGATCTGCCAAC-′;
Nrf2 reverse, 5′-ACGTAGCCGAAGAAACCTCA-′;
NOX1 forward, 5′-CTGGGTGGTTAACCACTGGTTT-′;
NOX1 reverse, 5′-ACCAATGCCGTGAATCCCTAAG-′;

Western blot analysis

We performed whole cell protein extraction and quantification as previously described. The cell lysates with an equal amount of total protein (40 µg) were resolved in a sodium dodecyl sulfate polyacrylamide gel electrophoresis set up and then transferred to polyvinylidene difluoride membranes. The blots were blocked in 5% skimmed milk for 4 h and incubated with rabbit antihuman primary antibodies against GAPDH (1:5000), Nrf2 (1:2000), NF-κB p65 (1:1000), or NOX1 (1:1000) overnight at 4°C. Afterward, the membranes were washed in PBS and incubated with goat antirabbit horseradish peroxidase–conjugated secondary antibody (1:6000) for 1 h. The proteins of interest were visualized using an enhanced chemiluminescence (ECL) detection reagent by an ImageQuant LAS4000 chemiluminescence detection system. The images were analyzed using the Quantity One software.

Luciferase activity assay

JASPAR database (https://jaspar.genereg.net/) was used to predict the Nrf2 potential transcription factor binding site in the NOX1 promoter region. The fragment in the 5′-flanking region of the NOX1 promoter (1500 bp) and that of the predicted binding site deletion mutant (1489 bp) was cloned, amplified, and inserted into pGL3 basic plasmid to construct recombinant plasmids; pGL3-NOX1-1500 and pGL3-NOX1-1489. The construction of pGL3-NOX1-1500 and pGL3-NOX1-1489 plasmids was identified using agarose gel electrophoresis. After transfection, the cells were stimulated by TNF-α, and the luciferase activity was monitored on an MD SpectraMax M5 enzyme–labeled instrument and was calculated as a ratio of firefly luciferase relative to Renilla luciferase luminescence.

Results

TNF-α upregulates NOX1 and Nrf2 expression in A549 cells

To examine the effect of TNF-α on the expression of NOX1 and Nrf2 in A549 cells, we profiled the expression of the NOX1 and Nrf2 in the A549 cells following TNF-α induction. Our data demonstrated that the expression of NOX1 and Nrf2 mRNA was increased with increasing concentrations of TNF-α, to a maximum of 10 ng/mL ($P < 0.05$) (Figures 1A and 1B). Similarly, the TNF-α significantly upregulated the levels of NOX1 and Nrf2, proportional to the incubation time, exerting its maximum effect at 24 h ($P < 0.05$). There were no statistical differences among the 24-, 36-m and 48-hour groups ($P > 0.05$). The A549 cells were cultured with 10 ng/mL of TNF-α for 24 h in subsequent experiments.

The efficiency of Nrf2 knockdown and Nrf2 overexpression

To test the effects of different vectors on the TNF-α-mediated expression of Nrf2 in the A549 cells, the mRNA and protein expression of Nrf2 in the A549 cells were assessed. The analysis showed that when compared with the naive group, there was suppression of the expression of the Nrf2 protein in the Nrf2 siRNA group and upregulation in the TNF-α-treated group ($P < 0.05$), negative control group ($P < 0.05$), as well in the empty vector group ($P < 0.05$) (Figures 1C and 1E). Notably, the expression of the Nrf2 was further increased in the cells harboring the Nrf2 overexpression ($P < 0.05$). There were no statistically significant differences among the TNF-α-treated group, negative control group, and empty vector group ($P > 0.05$).

Effects of Nrf2 on the expression of NF-κB in the TNF-α-induced A549 cells

NF-κB activation is an important indicator of oxidative stress. Next, we investigated the regulation of Nrf2 on the TNF-α-induced NF-κB expression in the A549 cells. The data revealed that when compared to the naïve group, the expression of NF-κB in the TNF-α-treated group ($P < 0.05$), negative control group ($P < 0.05$), and empty vector group ($P < 0.05$) significantly increased, with the highest level observed in the Nrf2 siRNA group ($P < 0.05$) (Figures 1D and 1E). Notably, the NF-κB level in the Nrf2 overexpression group was higher than that in the naïve group ($P < 0.05$) but lower than that in the TNF-α-treated group ($P < 0.05$), thus suggesting that Nrf2 could suppress the TNF-α-induced NF-κB expression.

Nrf2 reduced TNF-α-induced IL-6, IL8, ROS, MDA, or T-AOC production in A549 cells

To assess the effect of Nrf2 on TNF-α-induced inflammation and oxidative stress in the A549 cells, we quantified the levels of IL-6, IL8, ROS, MDA, or T-AOC. Compared to the naive group, there was an obvious increase in the levels of

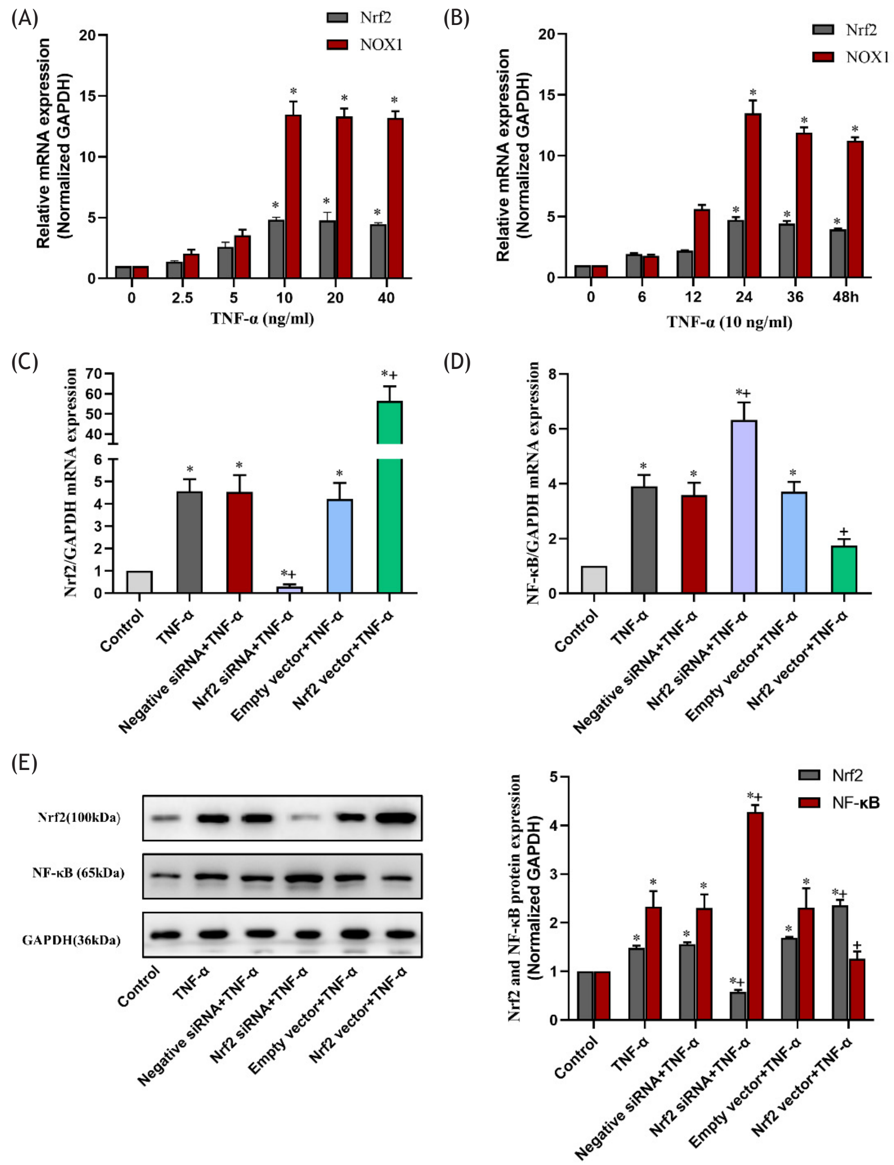

Figure 1 NOX1 and Nrf2 expression and the effects of Nrf2 on the expression of NF-κB in the TNF α induced A549 cells. (A) Nrf2 and NOX1 mRNA were induced by TNF-α at different concentrations for 24 h. (B) Nrf2 and NOX1 mRNA were induced by 10 ng/mL of TNF-α at different times. Control: A549 were cultured without siRNA or TNF-α; TNF-α: A549 were treated with TNF-α (10 ng/mL) for 24 h; Negative siRNA+TNF-α: A549 were pretransfected with Nrf2 siRNA and then treated with TNF-α (10 ng/mL) for 24 h; Nrf2 siRNA+TNF-α: A549 were pretransfected with Nrf2 siRNA and then treated with TNF-α (10 ng/mL) for 24 h; Empty vector + TNF-α: A549 were pretransfected with empty vector and then treated with TNF-α (10 ng/mL) for 24 h; Nrf2 vector + TNF-α: A549 were pretransfected with Nrf2 overexpression vector and then treated with TNF-α (10 ng/mL) for 24 h; (C) The expression levels of Nrf2 mRNA in different groups. (D) The effects of Nrf2 on the transcription of NF-κB (mRNA). (E) The effects of Nrf2 on the translation of NF-κB (protein). *P < 0.05, compared with the control group. #P < 0.05, compared with the TNF-α-treated group.

IL-6 and IL-8 in the TNF-α-treated group (P < 0.05), negative control group (P < 0.05), or empty vector group (P < 0.05), with higher elevation in the Nrf2 siRNA group (P < 0.05) (Figure 2A). Notably, the expression of IL-6 or IL8 in the Nrf2 overexpression group was lower compared to those in the TNF-α-treated group (P < 0.05) but higher than those in the naïve group (P < 0.05). Interestingly, the level of ROS (P < 0.05) and MDA (P < 0.05) correlated with the concentrations of IL-6 or IL-8 (Figure 2B). In contrast, compared with the naïve group, there was suppression of the T-AOC level in the TNF-α-treated group (P < 0.05), negative control group (P < 0.05), and empty vector group (P < 0.05), reaching its lowest in the Nrf2 siRNA group (Figure 2C). Besides, the T-AOC level in the Nrf2 overexpression group was lower compared to that in the naïve group (P < 0.05) but higher than that in the TNF-α-treated group (P < 0.05).

Nrf2 inhibited TNF-α-induced NOX1 expression in A549 cells via binding to the Nrf2 binding element in the NOX1 promoter

To test whether Nrf2 regulates the TNF-α-induced NOX1 expression in the A549 cells, we analyzed the NOX1

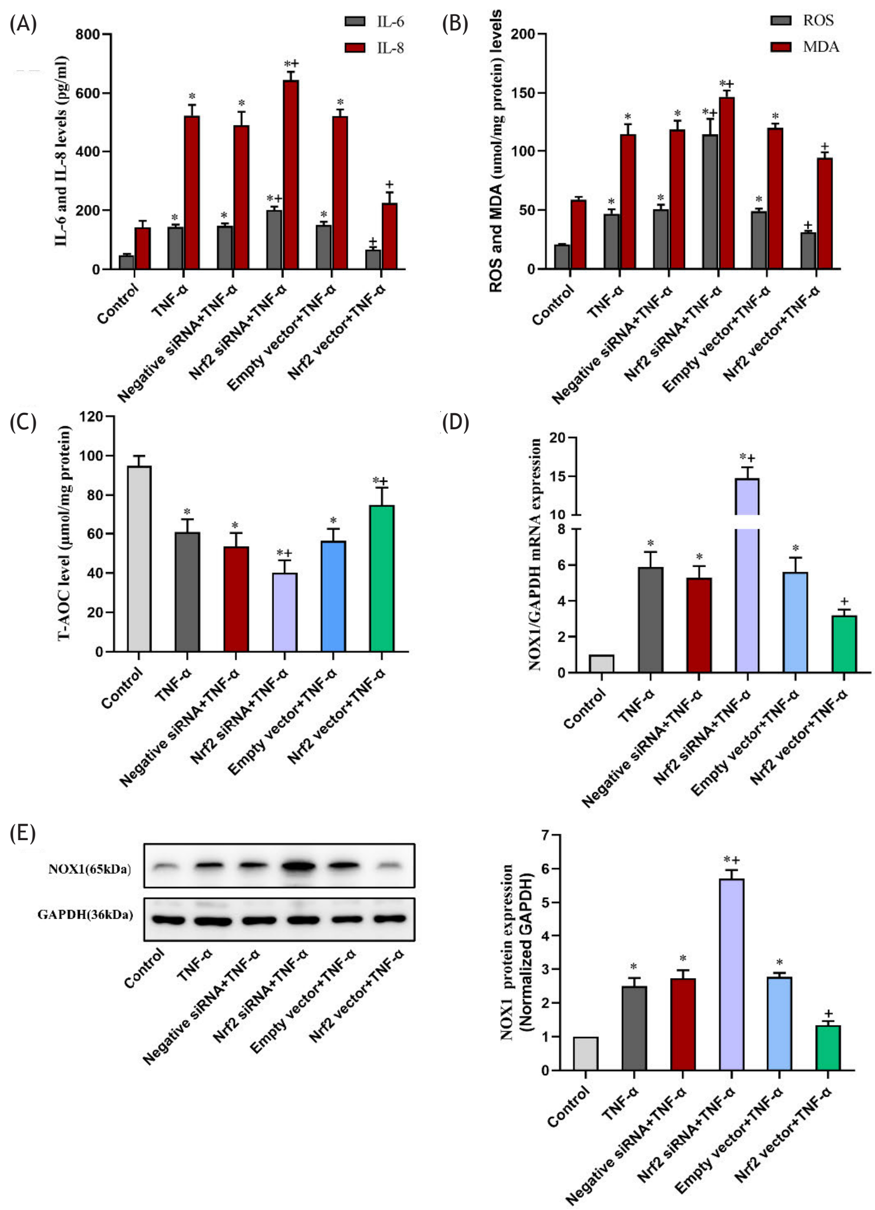

Figure 2 The effects of Nrf2 on the level of inflammatory reaction, oxidative stress, and NOX1. (A) The effects of Nrf2 on the production of IL-6 and IL-8. (B) The effects of Nrf2 on the production of ROS and MDA. (C) The effects of Nrf2 on the T-AOC level. (D) The effects of Nrf2 on the transcription of NOX1 (mRNA). (E) The effects of Nrf2 on the translation of NOX1 (protein). Control: A549 were cultured without siRNA or TNF-α; TNF-α: A549 were treated with TNF-α (10 ng/mL) for 24 h; Negative siRNA + TNF-α: A549 cells were pretransfected with Nrf2 siRNA and then treated with TNF-α (10 ng/mL) for 24 h; Nrf2 siRNA + TNF-α: A549 were pretransfected with Nrf2 siRNA and then treated with TNF-α (10 ng/mL) for 24 h; Empty vector + TNF-α: A549 were pretransfected with empty vector and then treated with TNF-α (10 ng/mL) for 24 h; Nrf2 vector + TNF-α: A549 were pretransfected with Nrf2 overexpression vector and then treated with TNF-α (10 ng/mL) for 24 h; *P < 0.05, compared with the control group. $^{\#}$P < 0.05, compared with the TNF-α-treated group.

expression profile in the A549 cells. The mRNA and protein levels of NOX1 were dramatically upregulated by TNF-α ($P < 0.05$), reaching their peaks in the Nrf2 siRNA group ($P < 0.05$), while those in the Nrf2 overexpression group were significantly suppressed ($P < 0.05$) (Figures 2D and 2E).

To further study the potential mechanisms by which the Nrf2 inhibits the expression of NOX1, the effect of the deletion of the Nrf2 binding element on the translational activity of the NOX1 promoter in the A549 cells was investigated. According to the JASPAR database online prediction website, there might be a strong targeting relationship between Nrf2 and NOX1. A potential Nrf2 binding site in the human NOX1 proximal promoter gene (1500 bp) was assessed: -1199/-1189 bp. The sequences of binding sites were as follows: 5'-ATTACACAGCA- 3'. (Figure 3A and Additional File 1). We cloned the NOX1 promoter and deleted the predicted Nrf2 binding element sequence. Agarose gel electrophoresis analysis showed that the

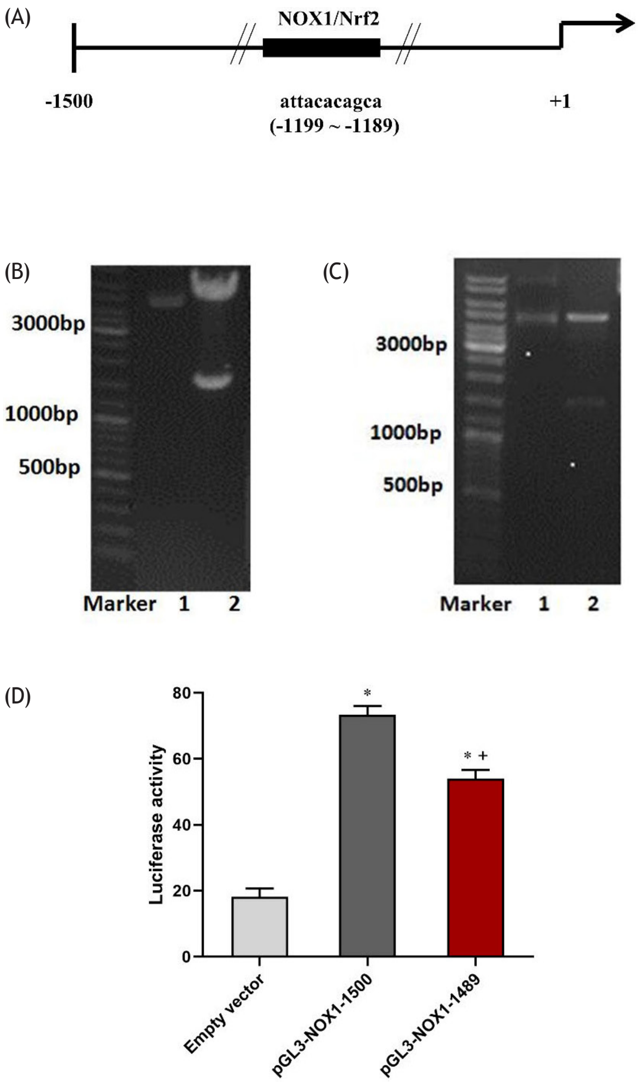

Figure 3 The effects of the Nrf2 binding element on the activity of NOX1 promoter. (A) Nrf2 binding element in the promoter of NOX1, the location of the binding sequence was counted relative to the ATG codon. (B) Agarose gel electrophoresis of pGL3-NOX1-1500 PCR products. Marker: DNA marker Fermentas SM0331; 1: pGL3 basic plasmid; 2: pGL3-NOX1-1500 plasmid digested by Kpn-I and Hind-III. (C) Agarose gel electrophoresis of pGL3-NOX1-1489 PCR products; Marker: DNA marker Fermentas SM0331; 1: pGL3 basic plasmid; 2: pGL3-NOX1-1489 plasmid digested by Kpn-I and Hind-III. (D) Luciferase activity in different groups. *P < 0.05, compared with the control group. $^{\#}$P < 0.05, compared with the TNF-α-treated group.

recombinant plasmid "pGL3-NOX1-1500" was a combination of pGL3 plasmid and full-length NOX1 promoter, while the "pGL3-NOX1-1489" plasmid was a combination of pGL3 basic plasmid and the deletion mutant (Figures 3B and 3C). The data demonstrated that the cells in the "pGL3-NOX1-1500" group had the highest luciferase activity, followed by those in the "pGL3-NOX1-1489" and pGL3 groups (P < 0.05) (Figure 3D). Collectively, our results suggested that Nrf2 could specifically bind to the -1199/-1189 bp (ATTACACAGCA) region of the NOX1 promoter and inhibit NOX1 expression in TNF-α-stimulated A549 cells.

Discussion

Severe inflammatory reactions and imbalanced oxidative stress are the major mediators of ALI pathogenesis and development in A549 cells.[6,19] Besides, NOX1 and Nrf2 are pivotal regulators of inflammatory reactions and oxidative stress levels. Here, we hypothesized that there could be cross talk between NOX1 and Nrf2 factors in ALI pathogenesis.

Uncontrolled inflammatory responses can cause abnormal production and accumulation of ROS in various structural cells, which might perturb the balance between oxidant and antioxidant cell defense systems, leading to oxidative damage or even death of structural cells.[20] NOX plays a significant role in many oxidative stress-associated pulmonary diseases, such as ALI.[21] Previous studies have reported oxidative stress damage in lungs from hypoxia-induced ALI mice. Besides, the reports showed that wild-type mice were more susceptible to developing severe pathological abnormalities, such as increased lung permeability, inflammation infiltration, and pulmonary epithelial damage, compared to their $NOX1^{-/-}$ counterparts.[22,23] Another study illustrated that TNFR-Fc fusion protein reduces oxidative stress damage by inhibiting the production of NOX1/ROS in the lungs in LPS-induced ALI.[24] In addition, the expression of NOX1 has been shown to be upregulated by TNF-α, leading to an excessive oxidative stress injury to alveolar epithelial cells.[22,25] In sync with this observation, our results showed that TNF-α markedly upregulates the expression of NOX1 in a concentration-dependent and time-dependent manner.

Due to its ability to suppress oxidative stress levels in alveolar epithelial cells, Nrf2 is a potent inhibitor of oxidative stress injury. Thus, it is one of the main cytoprotective mediators in alveolar epithelial cells with ALI.[11,12] Recent studies have reported that TNF-α induced IL-8 production in A549 cells and Nrf-2 inhibitor could increase IL-8 levels in A549 cells, supporting that Nrf-2 activity reduces IL-8 secretion.[26] However, no data that defines the underlying mechanism by which Nrf2 exerts its protective effects in TNF-α-induced A549 cells is available. Consistent with previous studies, our research indicated that overexpression of Nrf2 greatly reduced the production of IL-6, IL-8, ROS, and MDA, as well as increased the level of T-OAC.

Next, we explored whether Nrf2 regulation affects NOX1 in ALI. As expected, our study demonstrated that under the stimulation of TNF-α, with the increased expression of Nrf2, the transcription and translation activities of NOX1 were significantly reduced. Moreover, our study robustly demonstrated that the expression of Nrf2 and NOX1 were simultaneously upregulated by TNF-α induction, and there could be an Nrf2 binding element in the NOX1 promoter. Our data suggested that Nrf2 could inhibit the NOX1 transcriptional activity by binding to the -1199 to -1189 bp (ATTACACAGCA) region of the NOX1 promoter in TNF-α-stimulated A549 cells. This suggests that Nrf2 may regulate TNF-α-induced inflammatory response and unbalanced oxidative stress by inhibiting NOX1 transcriptional activity in the A549 cells. These findings indicated that NOX1 plays a central role in disrupting the equilibrium between the oxidant and antioxidant system of the A549

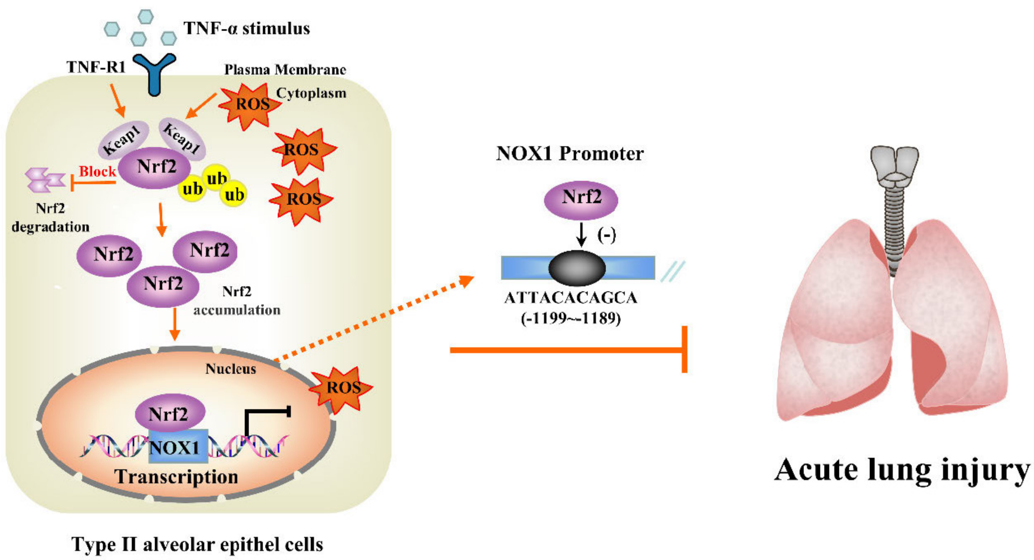

Figure 4 Schematic representation of the mechanism of Nrf2 on TNF-α-induced inflammatory reaction and oxidative stress via regulating NOX1 in alveolar epithelial type II cells. In TNFα-induced ALI, activated Nrf2 can translocate to the nucleus, where it binds to NOX1 promoter regions (ATTACACAGCA, -1199 ~ -1189) and suppress the transcription of NOX1, which can alleviate inflammatory and oxidative stress.

cells in ALI, and Nrf2 mediates the pathogenesis of ALI by downregulating the transcription and translation of NOX1.

Similarly, NF-κB acts as a nuclear transcription regulator and is closely associated with the pathogenesis of ALI.[27] More importantly, NF-κB has been shown to bind to the NOX1 promoter and regulates its expression,[28] a pathway that is mainly activated by TNF-α.[29] In addition, increasing evidence indicated that there is cross talk between Nrf2 and NF-κB.[30,31] Thus, NF-κB might participate in the downregulation of Nrf2, thus affecting the expression of NOX1 and the level of TNF-α-induced oxidative stress in A549 cells. The potential interplay between Nrf2 and NF-κB in the A549 cells was explored. Interestingly, our data showed that TNF-α significantly upregulated the expression of NF-κB, and the expression of NF-κB was inhibited by Nrf2.

In this study, TNF-α-induced A549 cells were used to explore the effects of the interaction between NOX1 and Nrf2 and their mechanisms on the pathogenesis of ALI. Our results indicated that Nrf2 might be mediating the downregulation of NOX1 by binding to the NOX1 promoter, thus exerting a significant reduction in the levels of IL-6, IL-8, ROS, and MDA, while increasing the T-OAC in the A549 cells with ALI. In addition, the expression of NF-κB was significantly inhibited by Nrf2, which might also contribute to the attenuation of TNF-α-induced oxidative damages in A549 cells. Nevertheless, our study did not fully investigate the mechanism of the interaction of Nrf2 and NOX1, as well as the roles that NF-κB play in the regulation of Nrf2 in the expression of NOX1. More studies are needed to ascertain the interplay between these factors and better understand the mechanism by which Nrf2 exerts the cytoprotective effect in ALI.

Conclusion

In this study, we found that Nrf2 can modulate TNF-α-induced ALI by regulating the inflammatory reaction, oxidative stress, and the expression of NOX1. Our data suggested that Nrf2 could inhibit the NOX1 transcriptional activity by binding to the -1199 to -1189 bp region of the NOX1 promoter in TNF-α-stimulated A549 cells (Figure 4). Our results provide a novel theoretical basis for future research on Nrf2 and the transcriptional mechanism of NOX1. Hence, Nrf2 or NOX1 might be a promising therapeutic target for clinical ALI therapy.

Competing Interests

The authors state that there are no conflicts of interest to disclose.

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Supplementary

A potential Nrf2 binding site in the human NOX1 proximal promoter gene

A potential Nrf2 binding site in the human NOX1 proximal promoter gene (1500 bp) was assessed using JASPAR database (https://jaspar.genereg.net/) (supplementary materials): -1199/-1189 bp. The specific results are as follows:

Matrix ID	Name	Score	Relative score	Sequence ID	Start	End	Strand	Predicted sequence
MA0150.1	MA0150.1.NFE2L2	12.009132	0.9001971520934955	seq1	322	332	+	ATTACACAGCA

-1520 aaacagaacc ctaagaatct aaaagcaaaa gaaaaaccaa tagaaagata gaccaaaaag
-1460 tagaataggc tcagaatagg ctcttttaaa aagagaaaac tcaaatggcc agcagttgaa
-1400 ttaaaagatg ctcaaactca ttagtaatca gggaaatgca aattaaaatc ataatacgat-1340 agttttccac acttacttga attataaaaa caaaaaagtc tggaaaatac caagggttgg
-1280 taagcatgta gaggaagtag aactctcatt cataactctc tgtagtatac atttaggtgg
-1220 tcacttcgga acgggtttgg a **attacacag** ca aagtagaa tatgtgcaaa tctcaggacc
-1160 ctggaatttt actcctgggt atatacctta gagaaactgt agcatatgtg tgacatttga
-1100 tcaacattgt tccatcatca tatccatcag tagtaggatg aatgaataca ttaatgtata
-1040 ttcattcatg caatggcata ttagatagca gtgtaagtga accgcaatta catgtacatg
-980 tatgaatctc aaaaacccaa tgttgaaaga agcaaaccac agaagcatac atacacactg
-920 ccaggtttca tttacaaaaa gttcaaaaac aggaaaaact aaacaatata ttgcttaggg
-860 atgcaattat agttagtaaa aatataaaga aaaataacag aatgattacc ccaaatttca
-800 ggatagtgat tacatccggt ggggtagagg aggggaagaa gatagatgtg atcagggagg
-740 gaaatacaaa gagctttaag atactggaga aaaatagtct attttcttta atctgagtag
-680 tgaacacata gatacttatt ccttaaaatt attctttaag ttacatatgt atgttttata
-620 tactcttctg tgtatatttc accattttag aaaagggaaa aaaaatcagt gcccagagct
-560 gaacacacaa ctctagtaaa tctatcatac tagaagacaa tcatctccat tcttttgagt
-500 gctctgcctc tgtttatttt gaaccaaagt gcacttttat acttgttaaa ttttctcttg
-440 ctctatttgg cccttctttt cacttgtcct tccagccagt caagttctcc ccaaagccat
-380 catcatatat gtcaaccaca gatcatcctc caggggaact ggtatgctaa agtttctgag
-320 ctagccaggc tgaaatccaa atggcagccg gcagatgtgg caacagtttg aaaagtgcac
-260 tttgaaacag cttccttacc acacacgctt ccctccctac ttctcctgaa gtaatctgtt
-200 tacagaccca gactaataat ctttttttatg agaaacttta gcaaatcttt tatctaggaa
-140 ggcaatgctt cacattaggt catgttgata agatgatgag agagaatatt ttcatccaag
-80 aatgttgcta tttcctgaag cagtaaaatc cccacaggta aaaccccttgt ggttctcata
-20 gatagggctg gtctatctaa gctgatagca cagttctgtc cagagaagga aggcagaata
41 aacttattca ttcccaggaa ctcttggggt aggtgtgtgt ttttcacatc ttaaaggctc
101 acagaccctg cgctggacaa atgttccatt cctgaaggac ctctccagaa tccggattgc