Nuclear erythroid 2 p45-related factor—2 Nrf2 ameliorates cigarette smoking-induced mucus overproduction in airway epithelium and mouse lungs

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Abstract

Background and objective: Nuclear erythroid 2 p45-related factor—2 (Nrf2) is known to play important roles in airway disorders, whereas little has been investigated about its direct role in airway mucus hypersecretion. The aim of this study is to determine whether this factor could protect pulmonary epithelium and mouse airway from cigarette-induced mucus overproduction.

Methods: Using genetic approaches, the role of Nrf2 on cigarette smoking extracts (CSE) induced MUC5AC expression was investigated in lung A549 cells. Nrf2 deficiency mice were smoked for various periods, and the airway inflammation and mucus production was characterized.

Results: Acute smoking exposure induced expression of MUC5AC and Nrf2 in both A549 cells and mouse lungs. Genetic ablation of Nrf2 augmented, whereas overexpression of this molecule ameliorated CSE-induced expression of MUC5AC. Nrf2 knockout mice, after exposure to cigarette smoking, displayed enhanced airway inflammation and mucus production.

Conclusion: Nrf2 negatively regulated smoking-induced mucus production \textit{in vitro} and \textit{in vivo}, suggesting therapeutic potentials of this factor in airway diseases with hypersecreted mucus.

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1. Introduction

Mucus overproduction and increased numbers of goblet cells represent cardinal features of various chronic productive airway diseases such as asthma, chronic obstructive pulmonary disease (COPD), and bronchiectasis. Excessive production of mucus obstructs airways by forming mucous plugs and results in recurrent endobronchial infections, which eventually contributes to morbidity and mortality in these diseases [1,2]. MUC5AC mucin is one of the most important types of airway mucus [3,4], and a variety of signaling pathways have been involved in regulation of this important mucin upon a variety of airway stimuli [5–8].

Cigarette smoke is one of the most commonly encountered risk factors of COPD development. The pathogenesis of COPD is not yet fully understood. Not only the imbalance between airway cellular protease and antiprotease but also that between oxidants and antioxidants may contribute to COPD development [9,10]. Cigarette smoke which contains thousands of oxidants can be divided into gaseous and particle phase. There are a large number of oxidants and free radicals in the gaseous phase, including carbon monoxide, acrylic...
aldehyde, hydrocyanic acid, nitric oxide, nitrogen dioxide, acetone, sulfide, ammonia and phenol, acetaldehyde and so on. The particle phase comprises different molecules of quinones, hydroquinones and semiquinones, which are oxidized derivatives of aromatic compounds with strong redox potential. All those molecules put huge oxidative stress on lung cells which destroy almost all the cellular components like lipids, proteins and DNA, thereby leading to cell apoptosis, participating in cell signaling, regulating immune responses and modulating cell proliferation [9–11].

The nuclear erythroid 2 p45-related factor-2 (transcription factor Nrf2) is recognized to play a key role in inducing antioxidant and detoxifying enzymes and cytoprotective proteins [12–14], which could protect cells and tissues against different oxidative insults such as hyperoxia [15], mechanical ventilation [16], cigarette smoking [17], inhaled allergen [18], virus [19], bacterial endotoxin [20], inflammatory factors [21], air pollution [22], and fibrogenic bleomycin [23]. When Nrf2 expression is reduced in the lung, damage and toxicity resulted from those harmful agents could be exacerbated.

Despite of the extensive studies of Nrf2 in lung disorders, little is known about the direct role of Nrf2 in airway mucus production. We have observed that lipopolysaccharide induced MUC5AC expression in a ROS dependent manner in lung epithelial cells [24]. Therefore, in the present study we attempted to investigate the effect of Nrf2 in cigarette smoking-induced mucus production in both lung epithelial cells and mouse lungs.

2. Methods

2.1. Cell culture

A549 cells were cultured in F12K medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 U/ml streptomycin (Gibco-BRL, Grand Island, NY) in a humidified atmosphere of 5% CO2 at 37 °C. Before experiments, confluent A549 cells were serum-starved for 24 h to maintain low basal levels of MUC5AC expression. Cigarette smoking extract (CSE) was prepared using 1 Kentucky research cigarette 3R4F (each has 9.4 mg tar and 0.726 mg nicotine) bubbled through 10 ml culture medium [25,26]. Cell viability was determined by the conventional 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, as described previously [25]. N-acetyl-l-cysteine (NAC) was product of Sigma.

2.2. Transfection of siRNA or cDNA

At 24 h after seeding, A549 cells were transfected with 100 nM of the Nrf2 siRNA (Invitrogen) or the scrambled RNA as a negative control (Invitrogen). For cDNA transfection, A549 cells were seeded for 24 h without antibiotics, and were then transfected with 1.0ug pcDNA-myc3-Nrf2 plasmid (Addgene, USA) and negative plasmid. Both siRNA and cDNA were transfected using Lipofectamin 2000 (Invitrogen), following the supplier’s protocols.

2.3. Animals

Six to eight week-old Nrf2−/− mice (ICR background) and their littermate controls were purchased from the Nanjing Pattern Animal Research Center, and the animal protocols and procedures were approved by the Ethical Committee for Animal Studies at Zhejiang University of China. Mice were exposed to cigarette smoke using a standard Teague smoking system (TE-10Z, Teague Enterprise, Woodland, CA, USA) for continuous 3, 7, or 14 days in an identified condition [25,26].

2.4. Assessment of mouse lung function

Twenty-four hours after the final smoking, unrestrained conscious mice were used for measurement of lung function in a barometric plethysmography system (Buxco, Troy, New York), as previously described [27]. Unrestrained conscious mice were placed in Buxco. Aerosolized normal saline (NS) or methacholine in increasing concentrations (6,12,25, and 50 mg/ml, Mch6-50) were nebulized through an inlet of the main chamber for 1 min, and readings were taken and averaged for 2 min after each nebulization. The main indicator of airway obstruction, measured as enhanced pause (Penh), shows a strong correlation with airway resistance measured using standard procedures [28].

2.5. Assessment of airway inflammation

Twenty four hours after the final smoking, mice were killed with a lethal dose of pentobarbital. Tracheas were cannulated, and bronchoalveolar lavage fluid (BALF) was recovered by flushing lungs with 0.8 mL phosphate-buffered saline (PBS). BALF cells were counted and spun onto glass slides using a cytopsin and stained with Wright's Giemsa. A total of 400 cells per slide were assessed, and differential cell counts were recorded based on the morphology and staining of the cells.

2.6. Lung histology

After BALF collection, the left lungs from different groups of mice were fixed in 10% buffered formalin, and embedded in paraffin. The lung tissue was stained with hematoxylin and eosin (HE) to visualize the cell recruitment and to assess the inflammatory situation. In addition, to detect airway mucus production, the lung was performed with periodic acid-Schiff (PAS) reagent (Sigma–Aldrich). All of the details are as previously described [27]. The histology sections were semi-quantified with Olympus microscope (10 × 20 magnification) for the inflammatory situation and mucus hypersecretion. All slides were examined in random blind fashion by two independent investigators.

2.7. Real-time PCR

Total RNA was isolated from cells and mice lung tissues using TRIzol Reagent (Invitrogen) according to the
manufacturer’s instruction. For RT-PCR, cDNA was generated by reverse transcription using 2 mg total RNA. The expression levels of Nrf2 and MUC5AC mRNA were determined by quantitative real-time PCR using the SYBR Green system (Takara) on a spectrofluorometric thermal cycler (iCycler; Bio-Rad).

2.8. Western blot analysis

Western blots were performed as described previously [25,26]. Briefly, western blots were performed using whole cell extracts, separated on 8–15% SDS–PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore, USA). The membrane was blocked with a solution of TBS containing 0.1% Tween 20 (TBS-T) and 5% nonfat milk. After three washes in TBS-T, the membrane was incubated in a dilution (1:1000 for Nrf2 and 1:200 for MUC5AC) of a primary antibody (ab24070 from Abcam for MUC5AC, ab76026 from Abcam and sc-722 from SantaCruz for Nrf2). After another three washes in TBS-T, the membrane was incubated with 1:10000 dilution of the corresponding secondary antibody. The membrane was reacted with chemiluminescence reagent ECL (Amersham Biosciences) to visualize to blots. If necessary, blots were quantified using ImageJ software (downloaded from NIH website).

2.9. ELISA

Levels of MUC5AC released into BALF were analyzed by ELISA using paired Abs (Wusheng Biotech., Shanghai, China) following the manufacturer’s instructions.

2.10. Statistical analysis

Data are presented as means ± SE. One-way ANOVA was used to determine statistically significant differences ($P < 0.05$).
3. Results

3.1. Induction of Nrf2 and MUC5AC by cigarette smoke extract (CSE) in A549 cells

As an initial approach towards determining the cytotoxicity of CSE, A549 cells were treated with various concentrations of CSE and the cell death were monitored by MTT assay. CSE at concentrations lower than 10% induced slight but insignificant cytotoxicity till 24 h, whereas it caused rapid cell death at concentrations higher than 10% (Fig. 1A). We therefore select 10% CSE for further mucus studies.

Treatment of A549 cells with 10% CSE time-dependently induced expression mRNA transcripts and proteins of MUC5AC (Fig. 2A). Further experiments demonstrated that Nrf2 knockdown significantly inhibited the CSE-induced expression of MUC5AC (Fig. 2B). Conversely, Nrf2 overexpression increased the mRNA and protein levels of MUC5AC (Fig. 2C). Treatment with N-acetylcysteine (NAC) also significantly suppressed CSE-induced expression of Nrf2 and MUC5AC (Fig. 2E).

Fig. 2. Effect of Nrf2 on CSE-induced expression of MUC5AC. (A–B) Effect of Nrf2 knockdown on the mRNA (A) and protein (B) levels of MUC5AC. *p < 0.05 compared with the corresponding controls. Blots from three independent experiments were quantified and shown in Fig. 2B. (C–D) Role of Nrf2 overexpression in the mRNA (C) and protein (B) levels of MUC5AC. *p < 0.05 compared with the corresponding control. Blots from three independent experiments were quantified and shown in Fig. 2D. (E) Effect of NAC on CSE-induced expression of Nrf2 and MUC5AC. *p < 0.05, **p < 0.01 compared with the corresponding controls without CSE treatment.
Fig. 3. Effect of Nrf2 on short-term smoking-induced airway inflammation. (A) Nrf2 deficiency increased the total cells, neutrophils, and lymphocytes in BALF. *p < 0.05. (B) Increased inflammation in Nrf2−/− mouse lungs. Lung sections were stained with H&E, and the levels of inflammation were semi-quantified. (C) Nrf2 deficiency further augmented the smoking-induced loss of lung function. *p < 0.05, **p < 0.01 compared with the corresponding wildtype controls. N = 8–10 for each group.
MUC5AC (Fig. 1B and C). Interestingly, this treatment also slightly induced expression of Nrf2 in both levels of mRNA and proteins (Fig. 1B and C), although the induction of Nrf2 was not as significant as that of MUC5AC.

3.2. Effect of Nrf2 on CSE-induced MUC5AC expression

We next sought to examine the direct role of Nrf2 in CSE-induced MUC5AC expression. Knockdown of Nrf2 by
siRNA further augmented the CSE-induced expression of both mRNA transcripts (Fig. 2A) and protein levels (Fig. 2B) of MUC5AC relative to the controls. On the contrary, over-expression of Nrf2 effectively ameliorated the MUC5AC expressions (Fig. 2C and D). Furthermore, NAC, a widely used antioxidant which is known to stimulate Nrf2 activation, significantly increased the mRNA levels of Nrf2 whereas markedly attenuated the expression of MUC5AC in a dose-dependent manner (Fig. 2E). These data demonstrated that Nrf2 could effectively inhibit CSE-induced MUC5AC expression.

3.3. Role of Nrf2 in smoking-induced airway inflammation and lung function in mice

We further explored the role of Nrf2 in smoking-induced acute airway inflammation and lung function in vivo. Exposure of wildtype control mice to cigarette smoke time-dependently induced a significant increase in the numbers of total cells, macrophages, neutrophils and lymphocytes (Fig. 3A). Interestingly, the numbers of total cells, neutrophils and lymphocytes were further increased in Nrf2−/− mice, whereas the numbers of macrophages were comparable between the Nrf2−/− mice and the control group (Fig. 3A). Histological analysis of the lung tissues also revealed that the Nrf2−/− mice displayed a more significant induction of inflammation (Fig. 3B).

At the 14th day of mice exposure to cigarette smoke, we also examined the lung functions between Nrf2−/− mice and their controls. Mice without smoke exposure exhibited same levels of lung functions between the two groups; however, after short exposure to smoke, the Nrf2−/− mice displayed more significant loss of lung function as compared to the control group (Fig. 3C).

3.4. Effect of Nrf2 on airway mucus production in vivo

Cigarette smoking slightly induced the expression of Nrf2 in wildtype mouse lung tissues (Fig. 4A), which was in agreement with the results in A549 cells that CSE treatment slightly induced Nrf2 levels (Fig. 1). As revealed by the PAS staining, cigarette smoking time-dependently induced mucus production in mouse airways, and the mucus production was again more significantly evidenced in the Nrf2−/− mice (Fig. 4B). Accordingly, the secreted levels of MUC5AC in BALF of Nrf2−/− mice were also significantly higher than those of wildtype controls (Fig. 4C).

4. Discussion

In the present study, we clearly demonstrated that acute cigarette smoking-induced expression of MUC5AC as well as the central antioxidant transcription factor Nrf2 in both alveolar epithelial A549 cells and mouse lungs, and the induction of Nrf2 in turn suppressed the mucus production (Fig. 5), as genetic ablation of Nrf2 augmented, whereas its over-expression ameliorated smoking-induced expression of MUC5AC both in vitro and in vivo.

As the key transcription factor responsible for the induction of cellular antioxidant enzymes and cytotoxic protective proteins, the role of Nrf2 in airway diseases has been widely investigated [15–24]. In all of these disease models, genetic disruption of Nrf2 led to enhanced disease conditions. More severe emphysema, more oxidized DNA compound, increased numbers of apoptotic cell numbers, more obvious bronchoalveolar inflammation, and significantly suppressed antioxidant and detoxifying enzyme expression could be observed in the lungs of Nrf2−/− mice compared to those in wildtype littermates after chronic exposure to cigarette smoke (4 or 6 months) [17,24]. Consistent with these findings, our current study demonstrated that acute cigarette smoking-induced rapid mucus production in the airways, which is also further augmented in the Nrf2−/− mice. Thus, the overall protective role of Nrf2 in cigarette smoking-induced emphysema and COPD should be due at least in part to its inhibitory effect on the mucus hyperproduction.

The exact mechanisms by which Nrf2 attenuates mucus production remain unclear and require further investigations. It is possible that Nrf2 might interrupt the expression of MUC5AC at transcription stages; however, it is most likely that Nrf2 exerts its inhibitory effect on mucus production though its up-regulation of antioxidant enzymes and subsequent down-regulation of ROS (Fig. 5). Indeed, Qi et al. have reported that Nrf2 inhibited neutrophil elastase induced MUC5AC expression through activation of antioxidant enzymes.
enzymes and reduction of ROS [29]. We have observed that ROS mediated lipopolysaccharide induced MUC5AC expression in lung epithelial cells [30]. NADPH oxidases such as Duox1 have also been demonstrated to play important roles in mucus production induced by various stimuli through generation of ROS [31]. We have recently observed that Duox1 and ROS mediated lipopolysaccharide induced mucus expression in human and mouse tracheal epithelial cells as well [32]. Apparently, Nrf2 mediated up-regulation of cellular antioxidant defenses mediated by Nrf2.

As shown in our research, the numbers of macrophages were comparable between the Nrf2−/− mice and the control group, while total cells, neutrophils and lymphocytes were increased in the Nrf2−/− mice. As the Nrf2−/− mice exhibit decreased antioxidant capacity, these mice should be more susceptible to cigarette smoking-induced lung injury. However, the reasons why the numbers of macrophages were not increased in the Nrf2−/− mice are not clear, but might due to the short-term exposure to cigarette.

It might be of interests to note that in multiple human studies, the levels of Nrf2 significantly decreased in lung tissues and in alveolar macrophages of those aged smokers and COPD patients [33–35], while levels of BACH1 and KEAP1, the spontaneous Nrf2 inactivators, were increased in patients with COPD [35]. In our study, we found that acute cigarette smoking even slightly induced Nrf2 expression. In agreement with our results, Tirumalai et al. have observed that in vitro exposure to acrolein, an electrophilic carcinogen in cigarette smoke, stimulated Nrf2 activation and transcriptional induction of phase 2 enzymes in type II lung cells [36]. Thus, the induction of Nrf2 by acute cigarette smoking may represent a spontaneous adaptive response evoked by the body to cope with the forthcoming oxidative damage, while chronic exposure of the lungs to smoke induces persistent and more formidable oxidative disaster which might overcome the antioxidant defenses mediated by Nrf2.

In summary, our present study demonstrated an important role of Nrf2 in suppression of acute cigarette smoking-induced mucus hyperproduction, which contributes to its overall protective effect in chronic airway diseases such as COPD and asthma.

Conflict of interest

The authors have no conflict of interest.

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