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Transcription Factor Nrf2 Plays a Pivotal Role in Protection against Elastase-Induced Pulmonary Inflammation and Emphysema

Yukio Ishii,1,2* Ken Itoh,†‡ Yuko Morishima,* Toru Kimura,* Takumi Kiwamoto,* Takashi Iizuka,* Ahmed E. Hegab,* Tomonori Hosoya,†‡ Akihiro Nomura,* Tohru Sakamoto,* Masayuki Yamamoto,†‡ and Kiyohisa Sekizawa*

Emphysema is one of the major pathological abnormalities associated with chronic obstructive pulmonary disease. The protease/antiprotease imbalance and inflammation resulting from oxidative stress have been attributed to the pathogenesis of emphysema. Nrf2 is believed to protect against oxidative tissue damage through the transcriptional activation of a battery of antioxidant enzymes. In this study, we investigated the protective role of Nrf2 in the development of emphysema using elastase-induced emphysema as our model system. We found that elastase-provoked emphysema was markedly exacerbated in Nrf2-knockout (KO) mice compared with wild-type mice. The severity of emphysema in Nrf2-KO mice correlated intimately with the degree of lung inflammation in the initial stage of elastase treatment. The highly inducible expression of antioxidant and antiprotease genes observed in wild-type alveolar macrophages was significantly attenuated in the lungs of Nrf2-KO mice. Interestingly, transplantation of wild-type bone marrow cells into Nrf2-KO mice retarded the development of initial lung inflammation and subsequent emphysema, and this improvement correlated well with the appearance of macropores expressing Nrf2-regulated antiprotease and antioxidant genes. Thus, Nrf2 appears to exert its protective effects through the transcriptional activation of antiprotease and antioxidant genes in alveolar macrophages. The Journal of Immunology, 2005, 175: 6968–6975.

Chronic obstructive pulmonary disease, including pulmonary emphysema, chronic airway obstruction, and chronic bronchitis, is a major public health problem predicted to emerge as one of the top five causes of death and disability worldwide by the year 2020 (1). Emphysema is pathologically characterized as a breakdown in alveolar architecture, with enlargement of alveolar airspaces due to the destruction of alveolar walls. Lung parenchymal inflammation, protease/antiprotease imbalance, and oxidative stress are thought to be important processes in the development of emphysema (2–4). These processes may interact or synergize with each other in provoking the characteristic pathological changes associated with emphysema.

Cigarette smoking is a major cause of emphysema. Importantly, however, not all smokers develop clinically significant emphysema, and this observation suggests that some additional factors may be involved in determining individual susceptibility to emphysema (5). A well-documented host factor is a hereditary deficiency of α1-antitrypsin, a major circulating inhibitor of serine protease that is synthesized in the liver (6, 7). This hereditary disease shows premature and accelerated development of panlobular emphysema in both smokers and nonsmokers. However, other candidate host factors influencing the risk of emphysema require identification because the majority of patients with emphysema have normal serum levels of α1-antitrypsin.

Nrf2 (NF-E2-related factor 2), a member of the cap’n’collar family of basic leucine zipper type transcription factors, has been shown to bind to the antioxidant response element (ARE)3 as an obligatory heterodimer with small Maf protein in the induction of phase 2 enzymes and antioxidant genes (8, 9). Nrf2 binds to the Kelch-like ECH-associated protein (Keap1), a cytosolic actin-binding protein, which retains it in the cytoplasm under normal physiological conditions. When cells encounter oxidative or xenobiotic stress, Nrf2 is released from Keap1, allowing its rapid translocation to the nucleus (10–13). Nrf2-knockout (Nrf2-KO) mice grow normally and are fertile, but are susceptible to oxidative stress and reactive electrophiles (14–18). Nrf2 plays essential roles in protection against pulmonary inflammation caused by environmental toxins such as butylated hydroxytoluene (19) and hyperoxia (20). Moreover, it is reported that Nrf2 regulates inflammation in carrageenan-induced pleurisy and acute lung injury (21, 22).

To understand the pathogenesis of emphysema, rodent models of elastase-inducible emphysema have been established (23, 24). Intratracheal instillation of porcine pancreatic elastase (PPE) first induces lung inflammation and subsequently results in alveolar wall destruction. These pathological changes closely mimic those in human emphysema. In the present study, we therefore investigated the susceptibility of Nrf2-KO mice to PPE-induced emphysema to explore the role of Nrf2 in protection against emphysema.

3 Abbreviations used in this paper: ARE, antioxidant response element; PPE, porcine pancreatic elastase; BAL, bronchoalveolar lavage; KO, knockout; SLPI, secretory leukoprotease inhibitor; BMT, bone marrow transplantation.
Materials and Methods

Animals and treatment

Wild-type BALB/c mice were purchased from Charles River Breeding Laboratories. Nr2-2-KO mice with a BALB/c background were established by specific deletion of the Nr2f gene segment (8). All mice used in this study were 6- to 8-week-old and maintained in our animal facilities under specific pathogen-free conditions. All animal studies were approved by the Institutional Review Board. The mice were anesthetized with fluothane (Takeda Pharmaceuticals) and given an intratracheal instillation of 25 μg of PPE (Elastin Products) in 0.1 ml of sterile saline solution or 0.1 ml of saline alone.

Lung histology and quantitation of emphysema

On days 1, 4, and 21 after PPE administration, mice were anesthetized by i.p. injection of pentobarbital (50 mg/kg body weight). The trachea and lung were removed together and inflated with 4% paraformaldehyde in PBS to a water pressure of 25 cm. The tissue was then embedded in paraffin and 2-μm thick sections were stained with H&E. Air space enlargement, was quantified by the mean linear intercept (Lm) in 20 randomly selected fields of tissue sections (25).

Bronchoalveolar lavage (BAL)

On days 1 and 4 after PPE administration, the lungs of terminally anesthetized mice were lavaged with six sequential 1-ml aliquots of PBS. The supernatant of the first BAL was used for analysis of albumin concentration (Sigma-Aldrich). The supernatant was also used for analysis of neutrophil elastase by spectrophotometrical measurement as previously described (26). Hemoglobin content in a pooled aliquot was represented as the absorbance at 412 nm. The remaining pool of BAL was centrifuged and resuspended in PBS. Cells were counted using a hemocytometer and a differential cell count was performed by standard light microscopic techniques based on staining with Diff-Quik (American Scientific Products).

Lung mechanics

Twenty-one days after PPE administration, terminally anesthetized mice were trachealized, and the trachea was cannulated. After opening the chest wall, the cannula was connected to a computer-controlled small animal ventilator (flexiVent; Scireq). The compliance was determined by recording the relaxation pressures during inflation and deflation in 0.1 ml steps between 0 and 25 cm H2O. Due to variation in the weight of each animal, the lung volumes were normalized by body weight. Pressures from the normalized compliance curves were then extrapolated at 0.1-ml increments and used to establish the mean static lung compliance in each group.

RNA blot analysis

Total RNA was extracted from the lungs of anesthetized mice using an RNasy Mini kit (Qiagen). Electrophoresis of RNA was conducted on formaldehyde-agarose gels and RNA was transferred to nylon membranes (Hybond-N+; Amersham Biosciences). Hybridization was performed using hybridization solution (ExpressHyb; BD Clontech) containing 10 × 106 cpm/ml [32P]-labeled mouse NADPH quinone oxidoreductase (NQO1), GST-Yc, heme oxygenase-1 (HO-1), peroxiredoxin 1 (Prxl), or GPDH cDNA fragments following the manufacturer's protocol. After washing, autoradiograms were made using a bioimaging analyzer (BAS5000; Fuji Photo Film).

Expression of neutrophil elastase and antiproteinase

Neutrophils were isolated from blood and BAL by standard Percoll density gradients. The presence of 2-ME and electrophoresed onto Immobilon membrane (Millipore). The membrane was subsequently probed by monoclonal anti-Nr2f Ab. For making mAb against mouse Nr2f, rats were immunized by GST-Nr2 fusion protein encoding an 140–318 of mouse Nr2f. As a loading control, the membrane was also probed with anti-lamin B Ab. To detect immunoreactive proteins, we used HRP-conjugated anti-rabbit IgG and ECL blotting reagents (Amersham).

Luciferase reporter gene assay

The 1.25 kb of the mouse SLPI 5′ regulatory region was amplified by PCR using mouse genomic DNA as template and the primers 5′-TGA GCA GCA CTA ACC TGA CTG CTG-3′ and 5′-AAG GGG AGC TCT GAT GAC CA-3′, then cloned into pgL3-basic vector (SLPI-luc). Deletion of the SLPI 5′ regulatory region was done by removing the SpeI fragment from SLPI-luc (ΔSpeI-luc). The murine macrophage cell line RAW 264.7 (American Type Culture Collection) was grown in DMEM (Invitrogen Life Technologies) containing 10% FBS and seeded in 6-well dishes 24 h before transfection. Cells were transfected with reporter and Nr2f expression plasmids (9) using Lipofectamine Plus reagent (Invitrogen Life Technologies) according to the manufacturer’s instructions. The luciferase assay was performed with the Dual-Luciferase Reporter Assay kit (Promega) following the supplier’s protocol and measured by a Biotum Luminometer (Berthold). Transfection efficiencies were routinely normalized to the activity of a cotransfected Renilla luciferase. The means of four independent experiments are shown with SE.

Immunocytochemistry

Paraffin sections (4-μm thick) were cut and mounted on poly-L-lysine coated glass slides. After removing the paraffin, endogenous peroxidases were quenched with 0.3% H2O2 in methanol. After washing, sections were incubated with anti-F4/80 Ab (Serotec), anti-HO-1 Ab (16), anti-Prxl Ab (17), or anti-SLPI (Santa Cruz Biotechnology) Ab and incubated for another hour with Histofine Simple Stain MAX-PO (Nichirei). Diaminobenzidine was used as a chromogen.

Bone marrow transplantation (BMT)

Femurs and tibiae were obtained from 6- to 8-week-old wild-type or Nr2f-KO mice. Bone marrow cells were harvested in RPMI 1640 medium and re-suspended in sterile PBS. Nr2f-KO recipient mice were irradiated at a dose of 9 Gy, followed by caudal vein injection of 20 × 106 cells. At the specified time points, the lungs of the recipient mice were lavaged and cells positive for F4/80 were sorted using FACS Vantage (BD Biosciences). DNA was extracted from the sorted cells and Nr2f gene expression was determined by PCR using the primers 5′-TGG AGG GGA GTA TTG AAG GCT GTG-3′ and 5′-GCC GCC TTT TCA GTA GAT GGA GG-3′. To determine the efficiency of donor cell influx to the recipient’s alveolar space, genome DNA was extracted from alveolar macrophages and the contribution of male cells to alveolar macrophages of female recipients was examined by real-time PCR as previously described (28). The male genomic DNA level was assessed through determining the SRY gene content in the sample by PCR. The results were normalized by β-actin gene content. Primer sequences are as follows: SRY, 5′-GGG TGC TTA GTG CTT TTC CCC GTC TGG GAC CTC GCT-3′ and 5′-GCC GCC TTT TCA GTA GAT GGA GG-3′; β-actin, 5′-CAG GGT GAT TCA CAC CCT CTG G-3′ and 5′-GCC ACA CTA CTT CTC ACC G-3′. Known amounts of male and female DNA were used to establish standard curves. Individual samples were normalized to the 100% male reference standard according to the following formula: 1.9 exp(Cp SRY) × exp(106 − Cp SRY)/1.9 exp(Cp β-actin) × exp(106 − Cp β-actin), where the threshold cycle Ct represents the crossing point for the samples of SRY or β-actin. The level of male DNA (SRY) was estimated by linear regression using all control mixture standards.

Statistics

Data were evaluated by ANOVA and Scheffe’s test. Values for p < 0.05 were considered to be statistically significant.

Results

Development of emphysema is enhanced in Nr2f-KO mice

To elucidate the role of Nr2f in protection against emphysema, we first evaluated the development of emphysema 21 days following PPE treatment. No pathological differences were observed between wild-type and Nr2f-KO mice before PPE treatment upon microscopic examination (Fig. 1, A and B). After PPE treatment,
enlargement of airspaces and alveolar wall destruction were observed in both wild-type (Fig. 1C) and Nrf2-KO mice (Fig. 1D). However, these pathological changes were much more severe in Nrf2-KO mice than in wild-type mice. As can be seen in Fig. 1D, the alveolar walls are more severely damaged in the lungs of Nrf2-KO mice treated with PPE compared with wild-type mice. To further characterize the development of emphysema in Nrf2-KO animals, we determined the airspace enlargement by measuring the mean linear intercept in 20 randomly selected fields from H&E stained tissue sections. The mean linear intercept values in Nrf2-KO mice were approximately three times higher than values found in wild-type mice after PPE treatment (Table I). A pulmonary function test revealed that static lung compliance was significantly increased in PPE-treated Nrf2-KO mice compared with compliance in saline-treated Nrf2-KO animals 21 days after treatment, showing very good agreement with histological observations (Fig. 1E). This increase in static lung compliance was not observed in wild-type mice following PPE treatment (Fig. 1E). Taken together, these histological and physiological evaluations demonstrate that Nrf2-KO animals are more susceptible to PPE-induced emphysema.

Table I. Mean linear intercept

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Linear Intercept (μm)</th>
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</thead>
<tbody>
<tr>
<td>Wild-type mice, saline-treated</td>
<td>58.2 ± 4.2</td>
</tr>
<tr>
<td>Nrf2-KO mice, saline-treated</td>
<td>56.7 ± 2.5</td>
</tr>
<tr>
<td>Wild-type mice, elastase-treated</td>
<td>86.7 ± 14.2</td>
</tr>
<tr>
<td>Nrf2-KO mice, elastase-treated</td>
<td>258.8 ± 15.9b</td>
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a Results are represented as mean ± SEM in each of five animals.
b Significantly different from other groups (p < 0.01).

To delineate the contribution of preceding lung inflammation to the development of emphysema, the severity of the initial hemorrhagic inflammation was compared between wild-type and Nrf2-KO mice 1 and 4 days after PPE treatment. In wild-type mice, hemorrhagic lung inflammation characterized by alveolar hemorrhage, alveolar edema, and the infiltration of neutrophils into airspaces was observed 1 day after PPE treatment (Fig. 2A). However, the hemorrhagic inflammation had resolved by day 4 following PPE treatment and emphysema had not developed by that time (Fig. 2B). In contrast, the pathological changes were much more severe in Nrf2-KO mice (Fig. 2C) than in wild-type mice by day 1 (Fig. 2A) and hemorrhagic lung inflammation persisted by day 4 (Fig. 2D). It should be noted that enlargement of the airspaces had already been observed in the lungs of Nrf2-KO mice by day 4 (Fig. 2D).

To quantify the enhancement of initial lung inflammation in Nrf2-KO mice, we analyzed several inflammatory parameters using BAL fluid. By day 1, the hemoglobin concentration in BAL fluid was significantly higher in Nrf2-KO mice than in wild-type mice (Fig. 3A). Although the hemoglobin concentration had decreased to control values in wild-type mice by day 4, the level of hemoglobin was still high in Nrf2-KO mice. Similarly, the albumin concentration, an indicator of lung vascular permeability, in BAL fluid was significantly higher in Nrf2-KO mice than in wild-type mice both 1 and 4 days after PPE treatment (Fig. 3B).

The number of neutrophils present in BAL fluid was also significantly greater in Nrf2-KO mice than in wild-type mice 1 day after PPE treatment (Fig. 3C). By day 4, the number of neutrophils remained high in Nrf2-KO mice, but recovered to a basal value in wild-type mice (Fig. 3C). Alveolar macrophages were induced by PPE treatment in both wild-type and Nrf2-KO mice (Fig. 3D). However, the induction was transient in wild-type mice and peaked at day 1, yet the induction persisted in Nrf2-KO mice. The neutrophil elastase activity in BAL fluid was significantly elevated in Nrf2-KO mice compared with that in wild-type mice both 1 and 4 days after PPE treatment (Fig. 3E). These results demonstrate that Nrf2-KO animals develop severe pulmonary inflammation and exhibit a higher protease activity in the airspaces in the early period following PPE treatment. Consequently, emphysema was apparent by day 4 in Nrf2-KO animals.

Neutrophil elastase activity in BAL fluids reflects the balance in elastase and anti-elastase activity in the airspaces. To clarify whether a high protease activity in Nrf2-KO mice is due to the enhanced expression of neutrophil elastase, its expression was evaluated in neutrophils obtained from blood or BAL fluids 1 day after PPE treatment in both wild-type and Nrf2-KO mice. In both genotypes of mice, only a few mRNA transcripts were expressed in blood neutrophils. Although neutrophils infiltrated into the airspaces by stimulation with PPE exhibited significantly higher expression of neutrophil elastase, expression was not different between wild-type and Nrf2-KO mice (Fig. 3F). Thus, a high protease activity in Nrf2-KO mice may not be due to increased elastase balance but due to decreased anti-elastase balance.

The inducible expression of host defense genes is attenuated in Nrf2-KO mice

To clarify whether a high protease activity in Nrf2-KO mice is due to the inactivation of antiprotease, we examined the expression of two antiprotease genes, a1-antitrypsin and SLPI. The constitutive expression level of a1-antitrypsin observed in both lung and liver was unaltered by PPE treatment and equivalent to that in Nrf2-KO
mice (Fig. 4A). The activity of serum α1-antitrypsin was consistent with this result (Fig. 4B). Interestingly, the SLPI gene was significantly induced in the lungs of wild-type mice 1 and 4 days after PPE treatment (Fig. 4C). In Nrf2−/− mice, however, SLPI mRNA was not induced by the stimulation of PPE (Fig. 4C). The expression of SLPI in the liver was unaltered by PPE treatment in both genotypes of mice (Fig. 4C). A database search revealed that several ARE consensus-like sequences exist in the 1.2-kb promoter region of the mouse SLPI gene (EMBL Accession no. AF205374). Cotransfection of the 1.2-kb SLPI promoter linked to a luciferase reporter gene and Nrf2 expression plasmid into RAW 264.7 macrophages revealed that the overexpression of Nrf2 activates reporter gene expression in a dose-dependent manner (Fig. 4D). Deletion of the region 950 bp upstream from the SLPI promoter that contains several potential AREs resulted in a marked decrease in Nrf2-mediated luciferase gene expression. These results indicated that the expression of SLPI is under Nrf2 regulation.

In addition to protease/antiprotease imbalance, oxidative stress is thought to be an important contributor to the pathogenesis of emphysema. Several studies have demonstrated that Nrf2 regulates the gene expression of a wide range of antioxidant enzymes. We therefore examined the induction of GST, NQO1, HO-1, and PrxI in the lungs of both wild-type and Nrf2−/− mice before and after PPE treatment. Small amounts of these mRNA transcripts were constitutively expressed in the lungs of both genotypes before PPE treatment. In wild-type mice, the expressions of PrxI and HO-1 were significantly elevated 1 and 4 days after PPE treatment (Fig. 5, A and B). In Nrf2−/− mice, however, these antioxidant enzymes were not observed to be up-regulated upon PPE treatment (Fig. 5, A and B). Comparably, the pulmonary expressions of GST-Yc and NQO1 were significantly increased in the lungs of wild-type mice, but not in Nrf2−/− mice, 1 and 4 days after PPE treatment (Fig. 5, C and D).

Nrf2-regulated antioxidant and antiprotease proteins are expressed in alveolar macrophages

To clarify which cell types express Nrf2-regulated genes, lungs were immunostained with anti-F4/80 (Fig. 6A and E), anti-PrxI (Fig. 6, B and F), anti-HO-1 (Fig. 6, C and G), and anti-SLPI Abs (Fig. 6, D and H). F4/80-positive macrophages were similarly detected in alveoli of both wild-type and Nrf2−/− mice (Fig. 6, A and E). In wild-type mice, macrophages were strongly stained with PrxI, HO-1, and SLPI 1 day after PPE treatment (Fig. 6, B–D). In contrast, no positive staining by these Abs was observed at any time point in the lungs of Nrf2−/− mice (Fig. 6, F–H and data not shown). To assess further whether Nrf2 is actually expressed in alveolar macrophages, we performed immunoblot analysis using anti-Nrf2 Ab and alveolar macrophage obtained from BAL. The result demonstrated that the expression of Nrf2 and its target gene HO-1 were induced in response to the Nrf2 activator diethylmaleate in wild-type alveolar macrophages, but not in Nrf2−/− alveolar macrophages (Fig. 6I).

Transplantation of Nrf2-positive bone marrow cells attenuated elastase-induced lung inflammation and emphysema in Nrf2−/− mice

The results thus far imply that alveolar macrophages are the main site of Nrf2-mediated gene expression. To further clarify the contribution of macrophages to Nrf2-mediated protection against elastase-induced lung damage, bone marrow cells from wild-type mice were transplanted into Nrf2−/− mice. We first examined the appearance of donor cells in the alveolar spaces of the recipient mice. BAL was performed on days 5, 14, and 35 after BMT and macrophages were isolated using a macrophage marker. The Nrf2 wild-type allele derived from BMT was detectable in these isolated cells by PCR 5 days after BMT and reached a saturated level by 14 days (Fig. 7A).

We then examined the effects of Nrf2 expression on the survival of macrophages. To this end, we used female wild-type mice as recipients and male Nrf2−/− or wild-type mice as donors, and examined the contribution of male cells to alveolar macrophages of female recipients using SRY gene as a marker (28). We found that significant numbers of donor cells actually flowed in the alveolar space of the lung of recipient mice without stimuli at day 14 after BMT. The contribution of male cells to alveolar macrophages of female recipients was 8.1% with wild-type mouse-derived bone marrow cells and 12.8% with Nrf2−/− mouse-derived bone marrow cells, showing that there was no significant difference in the
The influx of Nrf2-null donor cells from that of wild-type donor cells (Fig. 7B). These results demonstrate that Nrf2 does not influence the influx of macrophages from bone marrow cells without stimuli. Therefore, using this model system, we administered PPE 14 days after BMT.

Destruction of the alveolar architecture 21 days after PPE treatment was markedly less severe in mice transplanted with wild-type mouse bone marrow (Fig. 7C, left) than in mice transplanted with Nrf2-KO bone marrow (Fig. 7C, right). The degree of initial lung inflammation was also attenuated in mice transplanted with wild-type bone marrow, compared with mice transplanted with Nrf2-KO bone marrow. The BAL hemoglobin level (Fig. 7D, left), albumin concentration (Fig. 7D, center), and neutrophil number (Fig. 7D, right) were significantly decreased 1 day after PPE treatment in mice transplanted with wild-type bone marrow, compared with mice transplanted with Nrf2-KO bone marrow. Correspondingly, Nrf2-regulated host defense proteins such as HO-1, PrxI, and SLPI were expressed in macrophages 1 day after PPE treatment in mice transplanted with wild-type mouse bone marrow (Fig. 7E). These results demonstrate that the Nrf2-mediated defense system works mainly in macrophages in the protection against PPE-induced emphysema.

**Discussion**

This study unveiled for the first time that Nrf2-KO mice are highly susceptible to PPE-induced emphysema. We found that lung inflammation in both the initial and subsequent stages of PPE-provoked emphysema was markedly exacerbated in Nrf2-KO mice. The expressions of several antioxidant enzyme genes, such as NQO1, GST-Yc, HO-1, and PrxI and the antiprotease gene SLPI were markedly induced by PPE treatment in wild-type lung. However, the inducible expression of these genes was largely abolished in Nrf2-KO mice.

Nrf2-positive macrophages were observed in alveolar macrophages in wild-type lung. In Nrf2-KO mice transplanted with wild-type mouse bone marrow, both initial lung inflammation and subsequent emphysema were significantly ameliorated with the appearance of Nrf2-positive macrophages. Thus, Nrf2 is a key regulator in the macrophage-mediated defense system against lung injury.

Elastase, especially PPE, has been used to provoke emphysema in animal models. Kinetic studies and analysis of mutant mice lacking inflammatory mediators have demonstrated that emphysema induced by a low dose of PPE, as is the case for our study,
is not due to the immediate proteolytic activity of PPE, but is mostly due to the subsequent inflammation caused by PPE (29–32). Thus, the susceptibility of Nrf2-KO mice to PPE-induced emphysema might be due to a decreased antiprotease activity against PPE itself or a decreased anti-inflammatory activity against PPE-induced inflammation, or both. The latter possibility is in close agreement with the observation that Nrf2-KO mice are highly sensitive to pulmonary inflammation caused by environmental toxins such as butylated hydroxytoluene (8) and hyperoxia (9).

In addition to parenchymal inflammation and oxidative stress, an imbalance between proteases and antiproteases in the lung is important in the pathogenesis of emphysema. Neutrophil elastase has been implicated as the critical protease responsible for lung destruction. In the present study, neutrophil elastase activity in BAL fluids was significantly higher in Nrf2-KO mice than in wild-type mice after PPE treatment, suggesting that an imbalance between protease and antiprotease levels is conspicuous in Nrf2-KO mice. This elevated neutrophil elastase activity in Nrf2-KO mice might be due to increased neutrophil infiltration or due to a decrease in antiprotease gene expression.

α1-Antitrypsin is the major antiprotease that inhibits a number of serine proteases such as neutrophil elastase. Individuals with a hereditary deficiency in α1-antitrypsin are known to develop progressive emphysema at a relatively early age (6, 7). However, both the expression of α1-antitrypsin in the liver and its activity in serum did not differ between wild-type and Nrf2-KO mice. Interestingly, whereas the SLPI gene was highly inducible in the lungs of wild-type mice after PPE treatment, induction of the SLPI gene did not occur in the lungs of Nrf2-KO mice.

SLPI is an 11.7-kDa mucosal secretory protein identified as a serine protease inhibitor and involved in protection against neutrophil elastase damage (33). Unlike α1-antitrypsin, SLPI is produced at the site of inflammation by several inflammatory stimuli including LPS (34), neutrophil elastase (35), and proinflammatory cytokines (36). SLPI, therefore, may participate in the first line of defense against protease damage at the inflamed site. In addition to the antiprotease activity, murine SLPI has been shown to attenuate inflammatory cytokine production in macrophages (37, 38). Moreover, human SLPI has been demonstrated to prevent activation of the inflammatory transcription factor NF-κB by inhibition of IκB degradation (39). Thus, reduced production of SLPI, which acts as both antiprotease and anti-inflammatory factor at the site of lung inflammation, may be an additional important factor that explains the susceptibility of Nrf2-KO mice to emphysema. To our knowledge, this is the first report demonstrating that the antiprotease gene is under Nrf2 regulation.

In the present study, induction of Nrf2 target genes, such as HO-1, Prxi, and SLPI, was observed in alveolar macrophages in wild-type mice after PPE treatment. This observation is consistent with a series of studies in which Nrf2 target genes, including HO-1 and Prxi, were induced abundantly in macrophages (16, 17). It has also been demonstrated that macrophages, as well as epithelial cells, are the major sources of SLPI (34, 38). Moreover, we demonstrated that the promoter activity of SLPI up-regulated by Nrf2 in the macrophage cell line RAW 264.7 (Fig. 4D). Because macrophages are the major source of reactive oxygen species and proteases, as well as inflammatory cytokines, it is reasonable to hypothesize that the aberrant gene expression in Nrf2-KO macrophages may cause an overwhelming inflammatory response.
and thus evoke lung injury. In Nrf2-KO mice transplanted with wild-type mouse bone marrow, both initial lung inflammation and subsequent emphysema were significantly attenuated with the appearance of Nrf2-positive macrophages. A defense system based on macrophages, therefore, is thought to be important in protection against lung injury.

Chronic obstructive pulmonary disease is a major cause of chronic morbidity and mortality worldwide, so an effective treatment is in great want. Recently, the anti-inflammatory activities of the Nrf2/ARE system have been widely recognized (40). Thus, activation of Nrf2 in alveolar macrophages might be a useful therapeutic approach for protection against the development of lung inflammation and emphysema.

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Disclosures
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