Nrf2-deficient mice are highly susceptible to cigarette smoke-induced emphysema

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Inflammation, protease/anti-protease imbalance and oxidative stress play important roles in the pathogenesis of emphysema. Nrf2 counteracts oxidative tissue damage and inflammation through transcriptional activation via the anti-oxidant responsive element (ARE). To clarify the protective role of Nrf2 in the development of emphysema, the susceptibility of Nrf2-knockout mice to cigarette smoke (CS)-induced emphysema was examined. In Nrf2-knockout mice, emphysema was first observed at 8 weeks and exacerbated by 16 weeks following CS-exposure, whereas no pathological abnormalities were observed in wild-type mice. Neutrophilic lung inflammation and permeability lung damage were significantly enhanced in Nrf2-knockout mice 8 weeks after CS-exposure. Importantly, neutrophil elastase activity in bronchoalveolar lavage fluids was markedly higher in Nrf2-knockout mice preceding the pronounced neutrophil accumulation. The expression of secretory leukoprotease inhibitor, a potent inhibitor of neutrophil elastase, was inducible in wild-type, but not in Nrf2-knockout mice. This protease/anti-protease imbalance, together with the lack of inducible expression of ARE-regulated anti-oxidant/anti-inflammatory genes, may explain the predisposition of Nrf2-knockout mice to neutrophilic inflammation. Indeed, specific activators of Nrf2 induced the expression of the SLPI gene in macrophages. These results indicate that Nrf2 protects against the development of emphysema by regulating not only the oxidant/anti-oxidant balance, but also inflammation and the protease/anti-protease balance.

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of chronic morbidity and mortality that is anticipated to rank fifth in 2020 as a worldwide burden of disease according to the study published by the World Bank/World Health Organization (Murray & Lopez 1996). Pulmonary emphysema is one of the major manifestations associated with COPD and is pathologically characterized as a breakdown in alveolar architecture, with enlargement of alveolar airspaces due to the destruction of alveolar walls (Snider 1992; Nagai et al. 1994). Emphysema is also characterized by accumulation of inflammatory cells such as macrophages and neutrophils in the airways and lung parenchyma.

Cigarette smoking is the major risk factor for the development of emphysema (Viegi et al. 2001). A protease/anti-protease imbalance triggered by cigarette smoke (CS)-induced inflammatory cell infiltration has long been thought critical in the pathogenesis of emphysema (Turino 2002). In mouse models of CS-induced emphysema, deletion of the macrophage protease matrix metalloprotease 12 (MMP-12) gene or neutrophil elastase (NE) gene confers marked protection against CS-induced emphysema (Hautamaki et al. 1997; Shapiro et al. 2003). Oxidative stress, which is defined as a shift in the balance between cellular oxidants and anti-oxidants in favor of oxidants, is
thought to be one of the important processes in the pathogenesis of CS-induced emphysema (Repine et al. 1997; MacNee & Rahman 1999). In addition to directly damaging the lung, oxidative stress contributes to the protease/anti-protease imbalance by inactivating α-1 anti-trypsin (MacNee & Rahman 2001). Oxidants also promote inflammation, for example by activating the redox-sensitive transcription factor NF-κB, which orchestrates the expression of multiple inflammatory genes important in the pathogenesis of COPD, such as interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) (Barnes 2000).

In humans, however, only certain cigarette smokers develop clinically significant emphysema. This observation suggests that some genetic factors and other intrinsic factors that act to protect against inflammatory cell proteases or CS-induced inflammation may be involved in determining an individual’s susceptibility to emphysema (Siafakas & Tzortzaki 2002). The well-documented host factor α-1 anti-trypsin is a major circulating elastase inhibitor that is synthesized in the liver (Turino et al. 1969; Hubbard & Crystal 1991). Congenital deficiency of α-1 anti-trypsin causes premature development of emphysema, especially in smokers (McElvaney & Crystal 1997). However, since the majority of patients with emphysema have normal serum levels of α-1 anti-trypsin, other anti-proteases such as secretory leukoprotease inhibitor (SLPI), elafin and human elastase inhibitor have been considered as determinants of the susceptibility to emphysema. These protease inhibitors are considerably smaller molecules than α-1 anti-trypsin (e.g. SLPI (12 kDa), elafin (6 kDa)) and are able to inhibit proteinases in areas such as intercellular spaces and intracellular clefts that are not accessible to the larger α-1 anti-trypsin molecule (Lucey et al. 1990). SLPI is equally as effective as α-1 anti-trypsin as an inhibitor of NE (Smith & Johnson 1985; Smith et al. 1986) and three times more potent in inhibiting neutrophil-mediated lung matrix destruction (Kramps et al. 1991). Indeed, administration of SLPI attenuated neutrophil elastase-induced emphysema (Rudolphus et al. 1991; Siafakas & Tzortzaki 2002).

Nrf2 is a member of the family of cap’n’collar basic leucine zipper transcription factors and has been identified as a pivotal factor in the coordinated induction of phase 2 detoxifying and anti-oxidant enzymes under the regulatory influence of the anti-oxidant response element (Itoh et al. 1997; Ishii et al. 2000). Nrf2 knockout (Nrf2–/–) mice grow normally and are fertile, but are susceptible to oxidative stress and reactive electrophiles (Ishii et al. 2002). Indeed, Nrf2 plays essential roles in protection against pulmonary inflammation caused by environmental toxins such as butylated hydroxytoluene (Chan & Kan 1999) and hyperoxia (Cho et al. 2002) and regulates inflammation in carrageenan-induced pleurisy (Itoh et al. 2004). The present study, therefore, was conducted to determine the role of Nrf2 in both the onset and progression of CS-induced emphysema using wild-type and Nrf2–/– mice chronically exposed to CS inhalation.

Results

Development of emphysema in Nrf2–/– mice

We first evaluated the susceptibility of Nrf2–/– mice to CS-induced emphysema. No pathological findings were observed in either wild-type or Nrf2–/– mice before (Fig. 1A,E) or 4 weeks after exposure to CS (Fig. 1B,F). In Nrf2–/– mice, enlargement of the airspaces and destruction of the alveolar walls were first observed 8 weeks after the initial exposure to CS (Fig. 1G), with more severe damage to the alveolar walls being seen 16 weeks after CS exposure (Fig. 1H). In contrast, emphysema was observed neither in wild-type mice even after 16 weeks of CS exposure nor in mice exposed to room air (Fig. 1C,D, Table 1). We then morphometrically quantified the airspace enlargement in tissue sections prior to and 8 and 16 weeks after CS exposure. The mean values of the mean linear intercept in Nrf2–/– mice were significantly greater than those in wild-type mice both 8 and 16 weeks after CS exposure (Table 1). Similarly, the density of the alveolar surface area per unit volume of lung parenchyma was significantly decreased in Nrf2–/– mice compared with wild-type mice both 8 and 16 weeks after CS exposure (Table 1). We further evaluated static lung compliance between wild-type and Nrf2–/– mice before and after CS exposure. The mean values of the static lung compliances of wild-type and Nrf2–/– mice before CS exposure were essentially the same (Table 1). A significant increase in the static lung compliance was noted in Nrf2–/– mice both 8 and 16 weeks after the CS exposure, an increase that was not observed in wild-type mice (Table 1). Taken together, these histological and physiological evaluations demonstrated that Nrf2–/– animals are more susceptible to CS-induced emphysema.

Enhancement of preceding lung inflammation in Nrf2–/– mice

Lung inflammation is thought to be an important process in the pathogenesis of emphysema. To delineate the contribution of lung inflammation to the development of emphysema in Nrf2–/– mice, we analyzed several inflammatory parameters using bronchoalveolar lavage BAL fluids. An increased number of total cells and...
Macrophages in the BAL of both wild-type and Nrf2−/− mice was detected 4 and 8 weeks after CS exposure and was not significantly different between the two genotypes (Fig. 2A,B). The number of neutrophils present in the BAL fluids was significantly greater in Nrf2−/− mice than in wild-type mice 8 weeks after CS exposure (Fig. 2C). The albumin concentration, an indicator of lung vascular permeability, in the BAL fluids was also significantly higher in Nrf2−/− mice compared to wild-type mice 8 weeks after CS exposure (Fig. 2D). These results demonstrate that Nrf2−/− animals develop severe pulmonary inflammation upon exposure to CS.

To clarify the molecular mechanisms governing the enhanced inflammation observed in Nrf2−/− mice, the concentration of TNF-α was determined in the BAL fluids. Compared to unexposed mice, the concentration of TNF-α significantly increased 8 weeks after CS exposure to a similar extent in both wild-type and Nrf2−/− mice (Fig. 3A). Similarly, the concentration of macrophage inflammatory protein-2 (MIP-2), a mouse counterpart of IL-8, in the BAL fluids did not differ between the wild-type and Nrf2−/− mice (Fig. 3B).

**Attenuation of anti-oxidant/anti-inflammatory gene induction in Nrf2−/− mice**

Oxidative stress is thought to be important for the pathogenesis of emphysema. Several studies have...
demonstrated that Nrf2 regulates the gene expression of a wide range of anti-oxidant enzymes. We therefore examined the induction of heme oxygenase-1 (HO-1), peroxiredoxin I (PrxI), and NAD(P)H:quinone oxidoreductase 1 (NQO1) genes in the lungs of both wild-type and Nrf2–/– mice before and after CS exposure. In the lungs of wild-type mice, significant inductions of HO-1 (Fig. 4A), PrxI (Fig. 4B) and NQO1 (Fig. 4C) mRNAs were observed 4 and 8 weeks after CS exposure. In Nrf2–/– lungs, however, there was a lack of induction of these mRNAs in response to CS exposure (Fig. 4A–C).

**Table 1** Effect of chronic exposure to CS on lung measurements

<table>
<thead>
<tr>
<th>Time of exposure (weeks)</th>
<th>Genotypes</th>
<th>Mean linear intercept (µm)</th>
<th>Surface density (µm−1)</th>
<th>Static lung compliance (L/cm H2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>Wild-type</td>
<td>30.2 ± 2.0</td>
<td>0.121 ± 0.003</td>
<td>0.0786 ± 0.0019</td>
</tr>
<tr>
<td></td>
<td>Nrf2–/–</td>
<td>32.0 ± 0.7</td>
<td>0.120 ± 0.006</td>
<td>0.0656 ± 0.0048</td>
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<tr>
<td>8 weeks after:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>clean air exposure</td>
<td>Wild-type</td>
<td>31.0 ± 0.8</td>
<td>0.123 ± 0.002</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>Nrf2–/–</td>
<td>30.0 ± 0.6</td>
<td>0.113 ± 0.002</td>
<td>n.d.</td>
</tr>
<tr>
<td>CS exposure</td>
<td>Wild-type</td>
<td>35.7 ± 2.6</td>
<td>0.107 ± 0.014</td>
<td>0.0736 ± 0.0096</td>
</tr>
<tr>
<td></td>
<td>Nrf2–/–</td>
<td>48.7 ± 1.4*</td>
<td>0.092 ± 0.004*</td>
<td>0.1148 ± 0.0152*</td>
</tr>
<tr>
<td>16 weeks after:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>clean air exposure</td>
<td>Wild-type</td>
<td>30.2 ± 0.1</td>
<td>0.110 ± 0.004</td>
<td>0.0897 ± 0.0164</td>
</tr>
<tr>
<td></td>
<td>Nrf2–/–</td>
<td>31.5 ± 1.1</td>
<td>0.107 ± 0.005</td>
<td>0.0866 ± 0.0071</td>
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<tr>
<td>CS exposure</td>
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<td>0.110 ± 0.004</td>
<td>0.0784 ± 0.0039</td>
</tr>
<tr>
<td></td>
<td>Nrf2–/–</td>
<td>62.5 ± 0.4*</td>
<td>0.067 ± 0.012*</td>
<td>0.1171 ± 0.0070*</td>
</tr>
</tbody>
</table>

Data are expressed as the means ± SEM of six mice for each group. *Significantly different from time-matched wild-type mice (P < 0.05). n.d. stands for not determined.

**Figure 2** Enhanced inflammation in Nrf2−/− mice after CS exposure. The number of total cells (A), macrophages (B), neutrophils (C), and albumin concentration in the bronchoalveolar lavage (BAL) fluid of wild-type mice (■) and Nrf2−/− mice (□) before and 4 and 8 weeks after CS exposure. Data are expressed as the means ± SEM of six mice for each group. *Significantly different from time-matched wild-type mice (P < 0.05).
We next examined the degree of oxidative stress both in wild-type and Nrf2−/− mice before and after CS exposure. The plasma level of 8-isoprostane, a marker of oxidative stress, significantly increased 8 weeks after CS exposure in mice of both genotypes compared with that before CS exposure (Fig. 4D). However, at the 4-week time point, the level of 8-isoprostane was significantly increased only in Nrf2−/− mice (Fig. 4D). These results support our contention that oxidative stress was accumulated rapidly in Nrf2−/− mice. This result is consistent with the recent report that the level of 8-oxo-7, 8-dihydro-2′-deoxyguanosine, another marker of oxidative stress, is increased in the lungs of Nrf2−/− mice after CS exposure in comparison with wild-type mice (Rangasamy et al. 2004).

Impairment of phagocytosis of neutrophils by macrophages in Nrf2−/− mice

Recently, we found that the expression of the CD36 gene in macrophages is also under the influence of Nrf2 (Ishii et al. 2004). It was previously shown that an increased CD36 expression enhances phagocytosis of apoptotic neutrophils (Asada et al. 2004). Therefore, we next examined the phagocytosis of neutrophils by macrophages in both wild-type and Nrf2−/− mice before and after CS exposure. In wild-type mice, the number of macrophages that engulfed neutrophils was significantly increased 8 weeks after CS exposure compared with that before CS exposure. In Nrf2−/− mice, however, the...
number of macrophages that phagocytosed neutrophils was much lower than that in wild-type mice (Fig. 5A). These results indicated that macrophage-mediated clearance of neutrophils is impaired in Nrf2−/− mice.

Then, we assessed the expression of CD36 gene in both wild-type and Nrf2−/− mice before and after CS exposure. In wild-type mice, significant induction of CD36 mRNA was observed in macrophages 8 weeks after CS exposure (Fig. 5B). In Nrf2−/− mice, however, induction of the CD36 gene expression was not observed in the lungs following exposure to CS, suggesting that the decreased expression of CD36 is responsible for the reduced clearance of neutrophils in Nrf2−/− mice.

Enhanced protease/anti-protease imbalance in Nrf2−/− mice

A protease/anti-protease imbalance has been implicated in the pathogenesis of emphysema. We therefore examined the NE activity in the BAL fluids of both wild-type and Nrf2−/− mice. The NE activity was significantly elevated in Nrf2−/− mice compared to that in wild-type mice both 4 and 8 weeks after CS exposure (Fig. 6A). To elucidate the reason why the NE activity is increased in Nrf2−/− mice, we next examined the mRNA level for NE. In wild-type mice, the expression of the NE gene increased with the increase of neutrophils. In Nrf2−/− mice, NE gene expression was significantly increased 4 weeks after CS exposure compared with that before CS exposure, but was decreased by 8 weeks. However, the expression level of the NE gene was not significantly different between wild-type and Nrf2−/− mice at any time point (Fig. 6B). Therefore, we surmise that the difference of NE expression alone cannot explain the increased NE level in Nrf2−/− mice. We then examined the expression of the α-1 anti-trypsin gene in the lungs of both wild-type and Nrf2−/− mice. A similar level of induction of α-1 anti-trypsin gene expression was observed in the lungs of both genotypes after CS exposure (Fig. 6C). The activity of serum α-1 anti-trypsin was unaltered after CS exposure and was not different between wild-type and Nrf2−/− mice (Fig. 6D).

In addition to serine proteases such as NE, metalloproteinases, particularly MMP-12, are thought to be important in the pathogenesis of CS-induced emphysema. We therefore examined the expression of the MMP-12 gene in the lungs of both wild-type and Nrf2−/− mice. MMP-12 gene expression was elevated significantly after CS exposure in the lungs of mice of both genotypes, and was not different between them (Fig. 6E).

Inducible expression of the SLPI gene is attenuated in Nrf2−/− mice

SLPI is equally as effective as α-1 anti-trypsin as an inhibitor of NE (Smith & Johnson 1985; Smith et al. 1986). Therefore, we next examined the expression of the SLPI gene in the lungs of both wild-type and Nrf2−/− mice. RT-PCR analysis demonstrated that the expression of the SLPI gene was significantly induced in the lungs of wild-type mice after CS exposure, but not in the lungs of Nrf2−/− mice (Fig. 7A). We then examined the expression of SLPI in the lungs by immunocytochemical analysis. The results revealed that strong immunoreactivity against anti-SLPI antibody was observed both in macrophages and epithelial cells in the lung.
tissues of wild-type mice 8 weeks after CS exposure (Fig. 7B). However, no immunoreactivity was observed in the lungs of Nrf2−/− mice (Fig. 7C). The number of alveolar macrophages that were recognized by anti-F4/80 antibody was not different between wild-type and Nrf2−/− mice (data not shown). These results indicated that Nrf2-mediated induction of SLPI gene expression after exposure to CS occurs both in macrophages and epithelial cells.

To confirm that Nrf2 activation leads to the induction of SLPI gene expression, we examined the effect of Nrf2 inducers on the expression of the SLPI gene in the macrophage cell line RAW264.7. Previously, we demonstrated that Nrf2 is activated in macrophages by 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) during carrageenan-induced pleurisy (Itoh et al. 2004). In RAW264.7 cells, 15d-PGJ2, as well as the Nrf2 activators diethylmaleate and sulforaphane, induced the expression of the SLPI gene (Fig. 7D). In contrast, rosiglitazon and cigitazon, both of which are specific ligands for peroxisome proliferator-activated receptor-γ, did not substantially up-regulate the SLPI gene expression (Fig. 7D). These results, together with the lack of inducible expression of the SLPI gene in the lungs of Nrf2−/− mice, strongly argue that the expression of the SLPI gene is under the regulation of Nrf2.

**Discussion**

In the present study, mice lacking Nrf2 developed overt emphysema within 16 weeks of CS-exposure, while wild-type Balb/c mice were resistant to CS-induced emphysema. During the development of emphysema, NE activity in the BAL was significantly higher in Nrf2−/− mice. Furthermore, neutrophil accumulation in the BAL was increased, at least partly because of the decreased clearance of neutrophils. These results indicate
that Nrf2 protects against the development of emphysema by regulating not only the oxidant/anti-oxidant balance, but also inflammation and the protease/anti-protease balance.

Recently, Rangasamy et al. (2004) demonstrated that Nrf2 gene deletion enhanced the susceptibility of mice of an ICR/129sv mixed genetic background to CS-induced emphysema. Interestingly, however, the type of lung inflammation observed in the early course of emphysema was different from that observed in this study. In comparison with wild-type mice, an enhanced infiltration of macrophages in Nrf2-disrupted ICR mice was observed following exposure to CS. These cells may contribute to alveolar wall destruction through the activities of their elastolytic enzymes, particularly MMP-12.

In the present study, however, an enhanced infiltration of neutrophils, but not macrophages, was observed in the airspaces of Nrf2−/− mice with a Balb/c background in comparison to wild-type mice. Moreover, the expression level of the MMP-12 gene was not different between wild-type and Nrf2−/− mice during CS exposure in our model.

To clarify the molecular mechanism of the enhanced neutrophilic inflammation in Nrf2−/− mice, we examined the level of TNF-α and MIP-2 in BAL fluids. TNF-α, a representative pro-inflammatory cytokine produced by macrophages, has been reported to attract neutrophils both by direct and indirect mechanisms (Ming et al. 1987; Strider et al. 1990; Czermak et al. 1999). MIP-2 is a member of the CXC chemokine super-family and a known neutrophil chemotactic protein that corresponds to IL-8 in humans (Driscoll 1994). Although these proteins were elevated in the BAL fluids 8 weeks after CS exposure, the levels were not different between wild-type and Nrf2−/− mice, suggesting that the extent of neutrophil chemotaxis is similar in these mice. We therefore

**Figure 7** The regulation of SLPI gene expression by Nrf2 in macrophages. (A) SLPI mRNA expression in the lungs of wild-type mice (□) and Nrf2−/− mice (■) before and 4 and 8 weeks after CS exposure. For the quantification, the intensity of each band from RT-PCR analysis was measured and normalized to that of the GAPDH gene. (B, C) Immunocytochemical localization of SLPI in the lungs of (B) wild-type and (C) Nrf2−/− mice exposed to CS for 8 weeks. Alveolar macrophages (arrows) and epithelial cells (arrowheads) show positive staining for SLPI in wild-type mice. Asterisks in Nrf2−/− mice indicate nonspecific signals of erythrocytes in the capillaries. Bars: 20 μm. (D) Quantitative PCR analysis of SLPI gene expression in Raw264.7 cells treated with dimethyl sulfoxide (DMSO; lane 1), 15-deoxy-Delta12,14-prostaglandin J2 (15d-PGJ2; lane 2), diethylmaleate (DEM; lane 3), Sulforaphane (Sulf; lane 4), Rosiglitazone (Rosi; lane 5) or Ciglitazone (Cigli; lane 6). *Significantly different from time-matched wild-type mice (P < 0.05). †Significantly different from mice before exposure (P < 0.05). #Significantly different from DMSO-treated control mice (P < 0.05).
analyzed the expression of CD36, a scavenger receptor that is expressed in macrophages and mediates the clearance of apoptotic neutrophils (Asada et al. 2004). We recently demonstrated that Nrf2 transcriptionally regulates the CD36 gene expression in macrophages (Ishii et al. 2004). Our present study has demonstrated that the CD36 gene expression is not inducible in Nf2⁻/⁻ mice after CS exposure. Consistently, the number of macrophages that engulfed neutrophils was significantly lower in Nf2⁻/⁻ mice than in wild-type mice after CS exposure (Fig. 5A). An impaired neutrophil clearance from the inflamed site might be one reason for the enhancement of neutrophilic inflammation in Nf2⁻/⁻ mice.

It is also interesting that the NE activity was dramatically higher in the BAL fluids of Nf2⁻/⁻ mice 4 weeks after CS exposure. NE is a potent serine protease produced and secreted by neutrophils. A recent study using NE knockout mice demonstrated that NE significantly contributes to the development of CS-induced emphysema (Shapiro et al. 2003). The NE activity in BAL fluids reflects the balance in elastase and anti-elastase activity in the airspaces. The high elastase activity in Nf2⁻/⁻ mice may be due to reduced anti-elastase activity rather than to increased elastase activity, since the high elastase activity preceded the pronounced accumulation of neutrophils. Furthermore, the high elastase activity in Nf2⁻/⁻ mice coincided with a lack of inducible expression of the SLPI gene, indicating that the decreased expression of anti-protease contributes to the high NE activity in Nf2⁻/⁻ mice. In addition to directly inhibiting leukocyte-derived proteases (Abbinante-Nissen et al. 1993; Sallenave et al. 1994; Betsuyasu et al. 2002), SLPI suppresses the ongoing secretion of inflammatory mediators (Lentsch et al. 1999) and exerts anti-microbial activity (Lentsch et al. 1999; Lin et al. 2004). In fact, mice lacking SLPI are susceptible to LPS-induced endotoxic shock (Jin et al. 1997; Nakamura et al. 2003).

The present finding that SLPI gene expression is up-regulated by Nrf2 activators, such as 15d-PGJ₂, diethylmaleate and sulforaphane, indicates that the SLPI gene expression is under the influence of Nrf2. We found that the expression of the reporter gene linked with the 1.2-kb promoter and upstream sequences of the SLPI gene is activated by Nrf2 over-expressed in a transient transfection analysis in RAW264.7 cells (data not shown). As the 1.2-kb region of the SLPI gene contains several ARE sequences (GenBank AF205374), we speculate that the SLPI gene expression might be activated by direct binding of Nrf2 to ARE. A reduced production of SLPI, which acts as both an anti-protease and an anti-inflammatory factor at the site of lung inflammation, may represent an important factor in the susceptibility of Nf2⁻/⁻ mice to emphysema. Interestingly, Nrf2-dependent induction of the α-1 anti-trypsin gene occurred in the lungs of ICR mice upon CS exposure (Rangasamy et al. 2004), whereas the inducible expression of the α-1 anti-trypsin gene was not affected in Nf2⁻/⁻ mice in the present study. Nrf2-mediated regulation of anti-protease gene expression might also be different between the mouse strains.

It has been widely accepted that the protective effects of Nrf2 on oxidative tissue injury occur via ARE-mediated induction of anti-oxidant and phase 2 detoxifying enzymes (Itoh et al. 1997; Ishii et al. 2000, 2002). Previous studies demonstrated that Nf2⁻/⁻ mice show more severe acute lung injury and lower levels of lung mRNAs for anti-oxidant enzymes including NQO1, HO-1, and glutathione-S-transferase than wild-type mice when exposed to butylated hydroxytoluene (Chan & Kan 1999) and hyperoxia (Cho et al. 2002). In CS-induced emphysema, the expression of these anti-oxidant genes was significantly up-regulated in the lungs of wild-type mice, but not in the lungs of Nf2⁻/⁻ mice with an ICR background (Rangasamy et al. 2004), and similar results were obtained in the present study. It is also reported that the Nrf2 protein level and DNA-binding activity are increased by CS exposure (Rangasamy et al. 2004).

Cigarette smoke contains an array of oxidants, such as quinones and aldehydes, that are known to activate Nrf2 (Pryor & Stone 1993). Quinones are by themselves strong electrophiles and can undergo an active redox cycle that is capable of reducing molecular oxygen to generate superoxide. On the other hand, a recent study has demonstrated that acrolein, an unsaturated aldehyde found in cigarette smoke, induces Nrf2-mediated phase 2 enzyme gene expression (Tirumalai et al. 2002). Therefore, multiple components in cigarette smoke may contribute to the oxidative stress and hence to Nrf2 activation. In this study, we found that the plasma level of oxidized lipid 8-isoprostane was increased in Nf2⁻/⁻ mice at the 4-week time point, consistent with the report that the level of 8-oxo-7, 8-dihydro-2′-deoxyguanosine, another marker of oxidative stress, is increased in the lungs of Nf2⁻/⁻ mice upon CS exposure (Rangasamy et al. 2004). The enhanced oxidative stress in Nf2⁻/⁻ mice might contribute to the pathogenesis of emphysema through enhanced inflammation, decreased anti-protease activity, and enhanced apoptosis of lung cells.

In conclusion, the Nrf2-mediated defense system may act at several stages of inflammation, such as protease/anti-protease imbalance, neutrophil clearance, and protection from oxidative stress, all of which are thought to be important processes in the pathogenesis of COPD (Fig. 8). It is interesting that the type of enhanced inflammation seen in CS-exposed Nf2⁻/⁻ mice differs.
between these two mouse strains. This difference may have been due to the difference in Nrf2-target gene expression between the strains. Therefore, we speculate that, in humans, the Nrf2-mediated defense system varies from individual to individual. Since COPD is a major cause of chronic morbidity and mortality worldwide, there is great need of an effective treatment. Thus, activation of Nrf2 might be a useful therapeutic approach for protection against the development of COPD.

### Experimental procedures

#### Animals and exposure to cigarette smoke

Nrf2–/– mice of an ICR/129sv mixed genetic background (Itoh et al. 1997) were backcrossed with Balb/c mice for nine generations. Balb/c wild-type mice were purchased from Charles River Japan (Kanagawa, Japan). All the mice used in this study were 6–8 weeks old and maintained under specific pathogen-free conditions. All animal studies were approved by the Institutional Review Board of University of Tsukuba. Mice were exposed to the smoke from five cigarettes (Hilite: tar 19 ng/puff, nicotine 1.3 mg/puff; Japan Tobacco, Tokyo) in a sealed 7.5 L plastic exposure chamber for 50 min a day, 4 days a week for 16 weeks. Mice exposed to room air were used as controls.

### Lung histology and quantification of emphysema

The trachea and lung of terminally anesthetized mice were removed and inflated with 4% paraformaldehyde in PBS to a pressure of 25 cm H2O. The tissues were then embedded in paraffin and 2 μm-thick sections were stained with hematoxylin and eosin. Air space enlargement was quantified by the mean linear intercept in 20 randomly selected fields of tissue sections (Saetta et al. 1985). The density of the alveolar surface area per unit volume of lung parenchyma was also calculated as previously described (March et al. 1999).

### Bronchoalveolar lavage (BAL)

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 determining the albumin concentration (Sigma, St. Louis, MO, USA). The NE activity of the supernatant was measured by spectrophotometry at 405 nm using the synthetic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-paranitroanilide (Sakamaki et al. 1996). 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 microscopy based on staining with Diff-Quik (Baxter Scientific Products, McGaw Park, IL, USA).

### Lung mechanics

Terminally anesthetized mice were tracheostomized and the trachea was cannulated. After opening the chest wall, the cannula was connected to a computer-controlled small animal ventilator (flexiVent; Scireq, Montreal, Canada). 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.

### Measurement of α-1 anti-trypsin activity

The activity of α-1 anti-trypsin was calculated by determining the trypsin inhibitory capacity against bovine trypsin in the serum as previously described (Dietz et al. 1974).

### RT-PCR

Total RNA was extracted from the lungs of anesthetized mice using an RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA). The expressions of anti-oxidant, protease, and anti-protease genes were analyzed by RT-PCR using specific oligonucleotide primers (Table 2). The PCR products were analyzed in 1.5% agarose gel and the results were quantified by densitometry analysis using NIH imaging software. The gene
expressions were normalized to their respective GAPDH gene expressions.

**Measurement of cytokines**

The concentrations of TNF-α and MIP-2 in the supernatant of the first BAL fluid were determined by enzyme-linked immunosorbent assay according to the manufacturer's instructions (TNF-α: BioSource International, Camarillo, CA; MIP-2: R&D systems, Minneapolis, MN, USA).

**Measurement of 8-Isoprostane**

The plasma concentration of 8-Isoprostane(8-epi-PGF₂α) was determined with a competitive enzyme-linked immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA).

**SLPI gene expression in culture cells**

RAW 264.7 cells were stimulated with 5 μM 15d-PGJ₂, 100 μM diethylmaleate, 5 μM sulforaphane, 1 μM rosiglitazone, 50 μM ciglitazone, or dimethyl sulfoxide for 24 h. Total RNA was extracted and quantitative real-time PCR was performed using the following primers for SLPI: 5'-tgc caa gat gct tgc gtt ccaa-3' and 5'-tca cat cgg ggg cag gca gac ttt-3'. The gene expression level was normalized against the housekeeping gene 36B4 (Laborda 1991).

**Immunocytochemistry**

Lung sections from the mice exposed to CS for 8 weeks were reacted with anti-SLPI antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated for 30 min with Histofine Simple Stain MAX-PO (Nichirei, Tokyo). Diaminobenzidine was used as a chromogen.

**Statistical analysis**

Data were evaluated by analysis of variance and Scheffe's test. P-values < 0.05 were considered to be statistically significant.

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**References**


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