Trolox contributes to Nrf2-mediated protection of human and murine primary alveolar type II cells from injury by cigarette smoke

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Cigarette smoke (CS) is a main risk factor for chronic obstructive pulmonary disease (COPD). Oxidative stress induced by CS causes DNA and lung damage. Oxidant/antioxidant imbalance occurs in the distal air spaces of smokers and in patients with COPD. We studied the effect of oxidative stress generated by CS both in vivo and in vitro on murine primary alveolar type II (ATII) cells isolated from nuclear erythroid 2-related factor-2 (Nrf2)-/- mice. We determined human primary ATII cell injury by CS in vitro and analyzed ATII cells isolated from smoker and non-smoker lung donors ex vivo. We also studied whether trolox (water-soluble derivative of vitamin E) could protect murine and human ATII cells against CS-induced DNA damage and/or decrease injury. We analyzed oxidative stress by 4-hydroxynonenal expression, reactive oxygen species (ROS) generation by Amplex Red Hydrogen Peroxide Assay, Nrf2, heme oxygenase 1, p53 and P53-binding protein 1 (53BP1) expression by immunoblotting, Nrf2 nuclear translocation, Nrf2 and p53 DNA-binding activities, apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay and cytokine production by ELISA. We found that ATII cells isolated from Nrf2-/- mice are more susceptible to CS-induced oxidative DNA damage mediated by p53/53BP1 both in vivo and in vitro compared with wild-type mice. Therefore, Nrf2 activation is a key factor to protect ATII cells against injury by CS. Moreover, trolox abolished human ATII cell injury and decreased DNA damage induced by CS in vitro. Furthermore, we found higher inflammation and p53 mRNA expression by RT-PCR in ATII cells isolated from smoker lung donors in comparison with non-smokers ex vivo. Our results indicate that the Nrf2 and p53 cross talk in ATII cells affect the susceptibility of these cells to injury by CS. Trolox can protect against oxidative stress, genotoxicity and inflammation induced by CS through ROS scavenging mechanism, and serve as a potential antioxidant prevention strategy against oxidative injury of ATII cells in CS-related lung diseases.

COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; HO-1, heme oxygenase 1; NQO1, NAD(P)H:quinone oxidoreductase 1; ATII, alveolar type II cells; Nrf2, nuclear erythroid 2-related factor; ARE, antioxidant response element; HO-1, heme oxygenase 1; NQO1, NAD(P)H:quinone oxidoreductase 1; GCLc, gamma glutamyl-cysteine ligase catalytic subunit

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lipid peroxidation, and protects cellular structures against oxidative stress. Vitamin E is present in the lung and in alveolar type II (ATII) cells. Because of its ability to decrease oxidative stress and lessen inflammation, vitamin E has been evaluated to improve CS-associated diseases, for example, COPD and emphysema, lung tumors, asthma and cardiovascular disease. Trolox is a water-soluble vitamin E analog with a high capacity to capture ROS. It is used as a standard to check the antioxidant capacity of other molecules.

By decreasing oxidant insults to the lung, trolox could modulate the development of chronic lung diseases and lung function decrement. Moreover, higher antioxidant status is associated with lower risk of COPD. It has been reported that oxidant/antioxidant imbalance occurs in the distal air spaces and in alveolar wall cells of smokers and patients with COPD and emphysema. Therefore, in this study, we focused on human and murine primary ATII cells. As a novel approach, we isolated murine ATII cells from Nrf2 and Nrf2 mice, and purified them using magnetic MicroBeads as we recently reported. For the first time, we compared the effect of CS both in vitro and in vivo on injury of murine primary ATII cells isolated from Nrf2 and Nrf2 mice and human primary ATII cells in vitro. Furthermore, we analyzed freshly isolated human primary ATII cells obtained from smoker and non-smoker lung donors ex vivo. Our hypothesis was that Nrf2 activation is a key factor to protect ATII cells against oxidative injury by CS, and that trolox will abolish ATII cell injury, decrease DNA damage and inflammation induced by CS through a ROS scavenging mechanism.

**Results**

**Trolox and Nrf2 activation decrease inflammation induced by CS in vivo.** KC and IL-6 levels in bronchoalveolar lavage (BAL) were identified as inflammatory parameters after exposure of Nrf2 and Nrf2 mice to CS in vivo. Their levels were significantly increased in both genotypes exposed to CS in vivo in comparison with the control (Figure 1). However, KC and IL-6 levels were higher in BAL obtained from Nrf2 than Nrf2 mice. Treatment with trolox followed by exposure to CS completely abolished the inflammatory response induced by CS in Nrf2 mice. This compound also significantly decreased KC and IL-6 levels induced by CS in Nrf2 mice in comparison with CS alone. However, KC and IL-6 levels were still higher in comparison with control. Our results indicate that trolox abolishes inflammatory response induced by CS in Nrf2 mice and partially protects Nrf2 mice against inflammation in vivo.

**Trolox and Nrf2 activation protects murine ATII cells against injury induced by CS in vivo.** We analyzed protein expression in lung tissue obtained from Nrf2 and Nrf2 mice exposed to CS in vivo (Figure 2, Panel A). We compared oxidative stress using 4-hydroxynonenal (4-HNE), which is a product of lipid peroxidation. We found significantly higher Nrf2, NQO1, GCLc and 4-HNE levels in Nrf2 mice exposed to CS and their expressions were decreased by trolox administration. We also observed high expressions of p53 and 4-HNE induced by CS in Nrf2 mice, and their decrease by exposure to trolox followed by CS in vivo.

To determine the cell-specific expression of analyzed proteins, we isolated ATII cells from lung tissue of Nrf2 and Nrf2 mice exposed to CS in vivo (Figure 2, Panel B). We found significantly higher expressions of Nrf2, HO-1, NQO1, GCLc and 4-HNE induced by CS in ATII cells obtained from Nrf2 mice and lower levels of these proteins after trolox administration followed by CS. We observed enhanced expression of 53BP1, p53 and 4-HNE in ATII cells obtained from Nrf2 mice, and their levels were decreased by trolox administration followed by CS in vivo. Furthermore, NQO1 and GCLc expression in lung tissue and ATII cells obtained from Nrf2 mice exposed to CS and treated with trolox followed by CS in vivo correlate with Nrf2 levels. We did not detect their expression in Nrf2 mice.

We also determined Nrf2 nuclear translocation in murine lung tissue (Supplementary Figure 1). We did not observe Nrf2 translocation in wild-type mice after treatment with trolox, which is a ROS scavenger. However, we found significant Nrf2 translocation from the cytoplasm to the nucleus in lung tissue obtained from Nrf2 mice after exposure to CS, which indicates Nrf2 activation. Furthermore, this translocation was decreased after treatment with trolox followed by CS.
Figure 2. Trolox abolishes CS-induced injury and apoptosis in ATII cells obtained from Nrf2\(^{+/+}\) mice in vivo. Mice were treated with trolox for 5 days and exposed to CS for 4 days in vivo as described in the Materials and Methods section. Panel A—protein expression in lung tissue; Panel B—protein expression in ATII cells. a—Nrf2\(^{+/+}\) mice; b—Nrf2\(^{-/-}\) mice. Lane 1—control; lane 2—PBS; lane 3—trolox; lane 4—CS; lane 5—trolox + CS (immunoblotting). Relative expression of these proteins is also shown. Panel C—Paraffin-embedded lung sections were stained for proSP-C to identify ATII cells and with fluorescein to detect apoptotic cells by TUNEL assay. * Statistically significant difference compared with control \((P < 0.05)\). # Statistically significant decrease compared with CS alone.
exposure. This suggests a protective mechanism of trolox against oxidative stress. We did not detect Nrf2 in nuclear and cytoplasmic fractions obtained from Nrf2−/− mice.

CS also significantly increased the percentage of apoptotic ATII cells in Nrf2+/+ and Nrf2−/− mice in vivo (Figure 2, Panel C). We found that trolox provided partial protection for ATII cells isolated from Nrf2−/− mice and completely abolished apoptosis induced by CS in ATII cells obtained from Nrf2−/− mice. Our results indicate: (i) high susceptibility of ATII cells to injury induced by CS in vivo, (ii) higher susceptibility to DNA damage and oxidative stress of ATII cells isolated from Nrf2−/− than Nrf2+/+ mice and (iii) the protective effect of trolox and Nrf2 activation against ATII cell injury by CS in vivo.

Nrf2 activation and trolox abolishes CSE-induced murine ATII cell injury in vitro. As we observed murine ATII cell and lung injury induced by CS in vivo, we also wanted to determine the effect of CS extract (CSE)-induced ATII cell injury in vitro. We isolated and purified ATII cells from Nrf2+/+ and Nrf2−/− mice (Figure 3). We found that CSE induces Nrf2, HO-1 and p53 expressions in Nrf2+/+ mice. Furthermore, these protein levels were significantly decreased after treatment with 0.5 μM trolox. We detected significantly higher expressions of 53BP1, p53 and 4-HNE in ATII cells isolated from Nrf2−/− mice, which were decreased after treatment with trolox.

These results are consistent with data obtained from the induction of apoptosis by CSE in murine ATII cells in vitro as measured by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay (Figure 4). Apoptosis was higher in ATII cells obtained from Nrf2−/− in comparison with Nrf2+/+ mice. Furthermore, trolox significantly decreased the percentage of apoptotic ATII cells in both genotypes. However, this antioxidant compound only partially protected ATII cells isolated from Nrf2−/− mice against injury induced by CSE in vitro and completely abolished apoptosis in these cells obtained from Nrf2+/+ genotype.

We also compared inflammatory response in ATII cells isolated from Nrf2+/+ and Nrf2−/− mice after treatment with CSE in vitro (Figure 5). KC and IL-6 secretions were slightly but significantly increased by CSE only in ATII cells isolated from Nrf2−/− mice. Furthermore, treatment with trolox followed by CSE significantly decreased their levels compared with CSE alone. These results indicate higher susceptibility of ATII cells isolated from Nrf2−/− mice to DNA damage and injury in comparison with cells obtained from Nrf2+/+ mice. Furthermore, this suggests that trolox can protect ATII cells against injury presumably through ROS scavenging.

**Figure 3** Trolox decreases murine ATII cell injury induced by CSE in vitro. ATII cells were isolated from Nrf2+/+ (a) and Nrf2−/− (b) mice and purified as described in the Materials and Methods section. ATII cells were treated with 0.5 μM trolox for 24 h followed by exposure to 4% CSE for 24 h. Lane 1—control; lane 2—PBS; lane 3—trolox; lane 4—CSE; and lane 5—trolox+CSE (immunoblotting). Relative expression of these proteins is also shown. * Statistically significant increase compared with control (P<0.05). #Statistically significant decrease compared with CSE alone.
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activity, which can partially compensate the lack of antioxidant system regulated by Nrf2.

Trolox decreases human ATII cell injury induced by CSE in vitro. We isolated human ATII cells from lung donors to validate data obtained from murine ATII cells treated with CS both in vitro and in vivo. We found that trolox significantly decreased ROS generated by CSE in ATII cells in vitro as measured by Amplex Red Hydrogen Peroxide Assay (Figure 6, Panel A). These results indicate the protective role of trolox against ATII cell injury through ROS scavenging mechanism. We also found that treatment with trolox followed by CSE significantly decreased Nrf2, HO-1, p53, 53BP1 and 4-HNE expressions compared with CSE alone (Figure 6, Panel B).

The critical role of CSE on the mechanism of ATII cell injury was determined by p53 and Nrf2 DNA-binding activity using Transcription Factor Activation Array (Figure 6, Panel C). We found that ATII cell treatment with CSE in vitro leads to p53 and Nrf2 activation. Furthermore, treatment with trolox...
followed by exposure to CSE significantly decreased p53 and Nrf2 DNA-binding activity compared with CSE alone. This suggests a protective role of trolox against DNA damage and oxidative stress. These results are consistent with our immunoblotting data.

We also determined the inflammatory response in human ATII cells treated with trolox followed by CSE in vitro (Figure 6, Panel D). We found that trolox significantly decreased IL-8 and IL-6 levels induced by CSE as measured by ELISA. Our results indicate human ATII cell injury by CSE through ROS generation, oxidative stress, DNA damage and inflammatory response. These data are in agreement with our results obtained from murine ATII cells treated with trolox and CS both in vitro and in vivo.

Discussion

Cells respond to the accumulation of ROS generated by CS in two ways. The first one is focused on the antioxidant scavenging system through activation of Nrf2. While with the second, the cells respond to ROS-induced DNA damage through p53-mediated apoptosis in the case of unreparable DNA damage.27 In this study, we addressed these two responses in human and murine primary ATII cells exposed to CS in vitro, in vivo and ex vivo. We confirmed our hypothesis that Nrf2 activation is important to protect ATII cells against injury by CS. Furthermore, the antioxidant compound trolox decreased DNA damage and the inflammation process in ATII cells induced by CS through ROS scavenging mechanism. We show for the first time that trolox partially provides protection against ATII cell injury in the absence of Nrf2.

We found higher KC and IL-6 levels in BAL obtained from Nrf2−/− mice than from Nrf2+/+ mice exposed to CS in vivo. The discrepancy between the increase in Nrf2 not accompanied by an increase of HO-1 in lung tissue obtained from wild-type mice exposed to CS in vivo can be explained in three ways. First, it has been reported that HO-1 activation by Nrf2 represents a late event in the antioxidant response.28 Second, we used a low CS concentration (70 mg/m3) due to lethality in Nrf2−/− mice. HO-1 induction has been reported for wild-type mice when CS was used at much higher concentrations (up to 250 mg/m3).29 Third, we observed HO-1 induction in ATII cells but not in the lung tissue obtained from Nrf2−/− mice after exposure to CS in vivo. This suggests high ATII cell sensitivity to oxidative stress.
We also found that CS induces nuclear Nrf2 translocation \textit{in vivo}, which indicates its activation. We did not observe this translocation after trolox administration, which was also reported \textit{in vitro}.\textsuperscript{30} Nrf2 nuclear translocation was decreased after treatment with trolox followed by CS in comparison with CS alone, which suggests cytoprotective role of this antioxidant compound. These observations are in agreement with our previous studies in human alveolar type I-like cells treated with trolox followed by exposure to CSE \textit{in vitro}.\textsuperscript{31}

Furthermore, ATII cells isolated from Nrf2\textsuperscript{−/−} mice were more susceptible to injury by CS both \textit{in vivo} and \textit{in vitro}. Sussan \textit{et al.}\textsuperscript{32} reported lung injury in Nrf2\textsuperscript{−/−} mice by CS. However, to our knowledge, there are no data on \textit{in vivo} and \textit{in vitro} comparison in murine primary ATII cells with trolox supplementation. Chen \textit{et al.}\textsuperscript{33} reported that CSE induced apoptosis in mouse embryonic lung cells \textit{in vitro} as detected by comet assay. The involvement of p53 in the regulation of the redox homeostasis has been reported.\textsuperscript{34} High levels of p53 appear to be associated with the decrease of antioxidant defense and with the consequent increase of intracellular ROS.\textsuperscript{27} On the contrary, it was suggested that DNA damage-dependent activation of p53 also induces a significant accumulation of ROS followed by the induction of p53-dependent apoptosis.\textsuperscript{35} Our findings indicate the protective role of Nrf2 against the inflammatory process, oxidative stress-induced DNA damage and p53-dependent apoptosis induction by CS in murine ATII cells both \textit{in vivo} and \textit{in vitro}. 53BP1 is required for an appropriate cellular response to DNA damage; however, its function is not fully understood.\textsuperscript{4}

\begin{figure}
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\includegraphics[width=\textwidth]{figure7}
\caption{CS increases IL-8 and IL-6 levels in human primary ATII cells. Panel A—higher IL-8 (a) and IL-6 (b) levels in whole ATII cell extracts obtained from smoker in comparison with non-smoker lung donors as measured by ELISA. Panel B—smoking induces p53 mRNA expression in ATII cells isolated from smoker lung donors compared with non-smoker lung donors as measured by RT-PCR. * Statistically significant difference compared with non-smokers ($P<0.05$).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure8}
\caption{Trolox contributes to Nrf2-mediated protection of ATII cells from apoptosis, injury and inflammation induced by CS. CS generates oxidative stress, which is decreased by trolox and the activation of Nrf2. Lack of Nrf2 and this antioxidant compound leads to accumulation of ROS, induction of DNA damage, inflammation and p53-dependent apoptosis in ATII cells.}
\end{figure}
We observed higher 53BP1 levels in ATII cells obtained from Nrf2−/− mice than Nrf2+/+ mice exposed to CS. This suggests that 53BP1 is associated with CS, which reflects oxidative DNA damage due to the lack of the antioxidant defense system regulated by Nrf2.

Higher expressions of 53BP1 and p53 observed in murine lung tissue and ATII cells obtained from Nrf2−/− mice compared with Nrf2+/+ mice after exposure to CS in vitro or in vivo can be explained in two ways. First, these cells have higher susceptibility to DNA damage mediated by p53/53BP1 owing to the lack of antioxidant defense system. It has been recently reported that in murine embryonic fibroblasts obtained from Nrf2−/− mice, Mdm2 expression (p53 inhibitor) was repressed and p53 protein was highly accumulated compared with wild-type cells.36 Furthermore, cells with Nrf2 knockdown had a higher level of DNA strand breaks induced by nickel in vitro.37 Second, there is a cross-talk between Nrf2 and p53.38 Functional Nrf2 can induce antioxidant defense systems to prevent oxidative damage and p53 activation. This hypothesis can be also confirmed by the higher binding activity of Nrf2 than p53 after ATII cell exposure to CSE as detected by transcription factor DNA-binding activities (Figure 6). Further studies are required to complete our knowledge of the dominance or co-existence of these two pathways. Our results indicate that ATII cells isolated from Nrf2−/− mice and exposed to CS will require p53 activation: (i) to induce a compensatory increase in antioxidant proteins, which can counteract ROS attack on DNA, (ii) to repair DNA damage and/or (iii) to induce apoptosis for the prevention of conversion of acquired mutations to inherited mutations.36

We also analyzed the effect of supplementation with trolox, a ROS scavenger, against murine ATII cell injury followed by exposure to CS both in vivo and in vitro. This compound was able to ameliorate oxidative stress induced by CS as measured by 4-HNE levels. We also found that trolox significantly decreased KC and IL-6 levels, 53BP1 and p53 expression, and the percentage of ATII apoptotic cells measured by 4-HNE levels. We found that trolox decreased ROS generation, Nrf2, p53, 53BP1 and HO-1 expressions, and IL-8 and IL-6 levels induced by CSE. We have recently shown that trolox also protected human primary alveolar type I-like cells against injury induced by CSE in vitro.31 Moreover, Nardini et al.40 also showed that supplementation of human bronchial epithelial cells HBE1 with vitamin E strongly prevented acrolein-induced generation of intracellular oxidants. They concluded that the oxidative stress following acrolein exposure seems to be an important event triggering the apoptotic response in this model system. Furthermore, there are several clinical trials, which reported the importance of vitamin E against CS-induced lung injury. Intake of vitamin E was associated with reduced oxidant levels in current smoker COPD patients.46 Wu et al.47 also showed a reduction in oxidative-induced DNA damage of white blood cells in the vitamin E-supplemented group versus the placebo group after a 12-week supplementation period in COPD patients. Furthermore, assignment to vitamin E in healthy women led to a reduction in the risk of new chronic lung disease where CS was a strong predictor.23

In summary, our results indicate the susceptibility of murine and human ATII cells to injury by CS. Trolox can protect against oxidative stress, genotoxicity and inflammation induced by CS through a ROS scavenging mechanism (Figure 8). Further studies of the integration of Nrf2 and p53 signaling would be important to determine the cell fate in response to oxidative stress induced by CS and antioxidant prevention strategies in CS-related lung diseases.

Materials and Methods

Animals. We used wild-type C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA) and Nrf2−/− mice (C57BL/6 background, developed by Dr. M. Yamamoto48 and kindly provided by Steven Kleeberger, NIH/NIHES). Mouse colonies were maintained at National Jewish Health. All mice were fed ad libitum and housed in an Institutional Animal Care and Use Committee (IACUC)-accredited facility in HEPA-filtered cages. Animal care, handling and experimental procedures were carried out in accordance with a protocol approved by the IACUC of National Jewish Health. At 4 weeks of age, mice were fed an AIN-76A diet for 10 days before in vitro or in vivo experiments.

Nuclear and cytoplasmic fraction isolation. Lungs were harvested to isolate nuclear and cytoplasmic fractions for subsequent western blotting using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s recommendations. Briefly, tissue was homogenized in 10 μl/mg CER I buffer with a GentleMACS dissociator (Miltenyi Biotec Inc., Auburn, CA, USA). After that buffer CER II was added, the lysate was spun down and the supernatant was collected as the cytoplasmic fraction. The pellet was resuspended in buffer NER and vortexed for 15 s every 10 min for a total of 40 min. The lysate was spun down for 10 min at 20 000 × g and the supernatant was collected as the nuclear fraction. We used lamin-B1 and IKB-α, as markers of nuclear and cytoplasmic fractions, respectively, as previously reported.50

Murine ATII cell isolation and culture. Nrf2+/+ and Nrf2−/− mice were euthanized, and BAL fluid was obtained through a tracheal cannula as previously described.51 Mice were killed and lungs were perfused with PBS via the pulmonary artery.

Furthermore, these results indicate increased ATII cell injury, likely due to an impaired antioxidant defense system beyond the threshold of Nrf2 activity. Moreover, we also treated human ATII cells with trolox followed by exposure to CSE in vitro. We found that trolox decreased ROS generation, Nrf2, p53, 53BP1 and HO-1 expressions, and IL-8 and IL-6 levels induced by CSE. We have recently shown that trolox also protected human primary alveolar type I-like cells against injury induced by CSE in vitro.31
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ATII cells were isolated as we previously described.26 The trachea was exposed and cannulated, and dispace (BD Biosciences, Heidelberg, Germany) was instilled into the lung followed by low melting point agarose (International Biotechnologies Inc., New Haven, CT, USA) for 2 min. Lungs were then dissected, placed in a tube containing dispace for 5 min at 37°C and tissue was gently minced by GentleMACS Dissociator (Miltenyi Biotec Inc.). Unique to other studies, ATII cells were purified using CD45-negative depletion (CD45 MicroBeads; Miltenyi Biotec Inc.) and biotinylated anti-mouse Ep-CAM (e-Bioscience, San Diego, CA, USA) with Streptavidin MicroBeads (Miltenyi Biotec Inc.) for a positive selection. The purity of these cells as measured by flow cytometry using cytokeratin, and SP-A was > 90% (data not shown).26 Murine ATII cells were cultured as we previously described. Briefly, they were plated for 1 day in DMEM with 5% rat serum with 20 mM HEPES on micellic inserted coated with a mixture of 20% Engelbreth-Holm-Swarm tumor matrix (BD Biosciences) and 80% rat-tail collagen in DMEM and then cultured for 3 days with 1% CS-stripped FBS along with 10 ng/ml keratinocyte growth factor (R&D Systems Inc., Minneapolis, MN, USA).

Isolation and culture of human ATII cells. We obtained deidentified human lungs not suitable for transplantation and donated for medical research from the National Disease Research Interchange (Philadelphia, PA, USA) and the International Institute for the Advancement of Medicine (Edison, NJ, USA). We selected donors without a history of chronic lung disease and with reasonable gender, race, smoking history, cause of death, medical history and medications at the time of death. Smokers were individuals who smoked 10–25 cigarettes per day for 4 days using a Teague TE-10 smoking system (Teague Enterprises, Kentucky Tobacco Research and Development Center, Lexington, KY, USA). The average particulate matter was 70 mg/m³ and carbon monoxide levels were 300 p.p.m. The antioxidant, trolox (CAS 53188-07-1; Calbiochem–EMD Biosciences Inc., La Jolla, CA, USA), was dissolved in PBS. In some experiments, mice received 35 mg/kg body weight trolox in PBS by oral gavage every 24 h starting 1 day before exposure to CS and for 4 days during exposure to CS.

Treatment with trolox and CSE in vivo. Five-week-old mice were exposed to CS from Kentucky reference cigarette 3RF (University of Kentucky) for 5 h per day for 4 days using a Teague TE-10 smoking system (Teague Enterprises, Woodland, CA, USA). The average particulate matter was 70 mg/m³ and carbon monoxide levels were < 300 p.p.m. The antioxidant, trolox (CAS 53188-07-1; Calbiochem–EMD Biosciences Inc., La Jolla, CA, USA) was dissolved in PBS. In some experiments, mice received 35 mg/kg body weight trolox in PBS by oral gavage every 24 h starting 1 day before exposure to CS and for 4 days during exposure to CS.

With treatment with trolox and CSE in vitro. The CSE was prepared as we previously described. Briefly, the smoke of one 3RF cigarette without filter (Kentucky Tobacco Research and Development Center, Lexington, KY, USA) containing 9.5 mg tar and 0.72 mg nicotine was drawn into 12.5 ml DMEM with a peristaltic pump (Manostat 72-310-000; Barnant Company, Barrington, IL, USA). The pump was set at an optimum speed to allow one cigarette to burn in 15 min and resulting solution was considered 100% CSE. This solution was filtered and applied immediately to the murine (4%) and human (6%) ATII cell cultures for 24 h. In some experiments, ATII cells were treated with trolox for 24 h before exposure to CSE. Trolox concentration used in murine (0.5 μM) and human (10 μM) ATII cells in vitro was selected based on the viability assay using trypan blue staining and MTT assay (data not shown).

TUNEL assay. We analyzed the apoptosis of murine and human ATII cells using a combination of staining for anti-prosurfactant protein C (pro-SP-C; Chemicon, San Diego, CA, USA) and TUNEL (Promega, Madison, WI, USA) assay as previously described with slight modifications.55–57 Briefly, ATII cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in PBS and permeabilized with 0.2% triton X-100 (Sigma Chemicals Inc.). Then slides with cells were incubated for 1 h at 37°C in a humid chamber in the presence of Dtd. In the negative control, no Dtd was added whereas in the positive control 10 μM Dtnase (Promega) was used (data not shown), respectively, according to manufacturer’s recommendations. Cells were then blocked with a 1:1 mix of Superblock Blocking Buffer (Pierce, Rockford, IL, USA) and 3% normal donkey serum, and incubated with anti-proSP-C (Abcam, Cambridge, MA, USA, Ab-3786) for 1 h. Anti-rabbit secondary Alexa-Fluor 594 (Invitrogen Corp., Carlsbad, CA, USA) was added to slides and incubated for 30 min. Cells were mounted with Vectashield medium containing DAPI (Vector Laboratories, Burlingame, CA, USA) and analyzed by fluorescence microscopy (Zeiss Axioskop 2, Thornwood, NY, USA). The percentage of TUNEL-positive apoptotic ATII cells labeled with fluorescein (fluorescein-dUTP-labeled DNA) was calculated per 10 high-power fields (magnification 10 × 40).58

ELISA. IL-8, KC and IL-6 were measured by ELISA (ELISA Tech., Aurora, CO, USA) in the murine BAL, murine and human ATII cell culture supernatant or in extracts of freshly isolated human ATII cells. We followed the manufacturer’s recommendations using a MicroQuant microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) and data were analyzed with KCJunior Data Analysis Software (Biotek Instruments, Winooski, VT, USA).

Western blotting. Expression of proteins from ATII cells or lung tissue were measured by western blotting as we described previously. Briefly, polyclonal antibody to mouse CSE-A was run in tris glycin buffer to separate the proteins. Protein loading was normalized to mouse anti-α-glycerophosphate-3-phosphate dehydrogenase (anti-GAPDH) purchased from Abcam. Mouse anti-β-actin was purchased from Abcam. Rabbit anti-NFκB, anti-p53, anti-NQO1, anti-LC3 and anti-IKB-α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit anti-53BP1, rabbit anti-4-HNE and rabbit anti-GCLc from Abcam. Horseradish peroxidase (HRP)-conjugated AffiniPure donkey anti-rabbit IgG and HRP-conjugated AffiniPure donkey anti-mouse IgG were purchased from Jackson Immunoresearch (West Grove, PA, USA). The blots were then developed using an enhanced chemiluminescence western blotting kit according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Images were quantitated using NIH Image 1.62 software (Bethesda, MD, USA).

ROS production. We compared ROS production in human ATII cells treated with 10 μM trolox followed by 6% CSE in vitro. We used the Amplex Red Hydrogen Peroxide Assay (Invitrogen Corp.) as a quantitative index of ROS generation.59,60 Hydrogen peroxide is one of the most stable forms of ROS, therefore this method allows observation of oxidation processes in real time. Amplex Red reacts with hydrogen peroxide in the presence of HRP with a 1:1 stoichiometry to form resorufin. Briefly, 50 μl of samples and standards were mixed with 50 μl of 100 μM Amplex Red and 0.2 U/ml HRP solution, and incubated for 30 min. Absorbance was measured at 560 nm (SpectraxMax340PC, Molecular Devices, Sunnyvale, CA, USA) and calculated concentrations were normalized to protein content.

Transcription Factor Activation. Transcription factor DNA-binding activities were measured using Transcription Factor Activation Profiling Plate Array II (Signosis, Sunnyvale, CA, USA) in nuclear extracts prepared from human ATII cells according to manufacturer’s recommendations. Briefly, biotin-labeled probes were mixed with these extracts to allow formation of transcription factor–DNA complexes for 30 min at 16°C. After elution of bound probe, a hybridization plate was used for quantitative analysis. The captured transcription factor probe was detected with streptavidin–HRP conjugate and measured as relative light units on a microplate luminometer (SpectraxMax340PC, Molecular Devices).

Real-time qPCR. Total RNA was isolated from human ATII cells using the RNeasy Mini kit (Qiagen, Germantown, MD, USA) according to the manufacturers’ recommendations. Assay on-demand p53 probe (hs99999147_m1) was purchased from Life Technologies (Grand Island, NY, USA). Before reverse transcription, isolated RNA was quantified and quality with a Nanodrop 1000 spectrophotometer, and the 0.5 μg of RNA was used to synthesize cDNA using qScript CDNA Supermix (Quanta Biosciences, Gaithersburg, MD, USA) in a total sample volume of 30 μl. The cycling conditions for RT-qPCR were as follows: 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. Taqman Gene Expression Master Mix (Life Technologies) was then used for qPCR with a total sample volume of

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15 μL. Before use, hydrolysis probes were tested for efficiency and optimal annealing temperature. Gene expression levels were calculated as a ratio of the p53 probe to the expression of the reference gene, GAPDH (Hs9999991_m1, Life Technologies) using the C1000 Thermocycler (Bio-Rad, Hercules, CA, USA). Cycling conditions were 50 °C for 2 min, 95 °C for 10 min and then 39 cycles of amplification at 95 °C for 15 s, 60 °C for 1 min. Data were analyzed using the ΔΔCt method.

Statistical analysis. One-way ANOVA by GraphPad Prism 4 (La Jolla, CA, USA) was used. A Dunnett’s test was applied (P<0.05). Data are shown here as the mean ± S.E.M. from at least three independent experiments.

Conflict of Interest
The authors declare no conflict of interest.

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