Distal Airways in Mice Exposed to Cigarette Smoke

Nrf2-Regulated Genes Are Increased in Clara Cells

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Cigarette smoke (CS) is the main risk factor for chronic obstructive pulmonary disease (COPD). Terminal bronchioles are critical zones in the pathophysiology of COPD, but little is known about the cellular and molecular changes that occur in cells lining terminal bronchioles in response to CS. We subjected C57BL/6 mice to CS (6 d/wk, up to 6 mo), looked for morphologic changes lining the terminal bronchioles, and used laser capture microdissection to selectively isolate cells in terminal bronchioles to study gene expression. Morphologic and immunohistochemical analyses showed that Clara cell predominance remained despite 6 months of CS exposure. Since Clara cells have a role in protection against oxidative stress, we focused on the expression of antioxidant/detoxification genes using microarray analysis. Of the 35 antioxidant/detoxification genes with at least 2.5-fold increased expression in response to 6 months of CS exposure, 21 were NF-E2-related factor 2 (Nrf2)-regulated genes. Among these were cytochrome P450 1b1, glutathione reductase, thioredoxin reductase, and members of the glutathione S-transferase family, as well as Nrf2 itself. *In vitro* studies using immortalized murine Clara cells (C22) showed that CS induced the stabilization and nuclear translocation of Nrf2, which correlated with the induction of antioxidant and detoxification genes. Furthermore, decreasing Nrf2 expression by siRNA resulted in a corresponding decrease in CS-induced expression of several antioxidant and detoxification genes by C22 cells. These data suggest that the protective response by Clara cells to CS exposure is predominantly regulated by the transcription factor Nrf2.

Keywords: antioxidant; detoxifying enzymes; laser capture microdissection; oxidative stress

Chronic inhalation of cigarette smoke (CS) is the major risk factor for chronic obstructive pulmonary disease (COPD). However, little is known about the molecular and cellular events that lead to COPD. With growing evidence demonstrating increased oxidative stress in smokers, a role of oxidant/antioxidant imbalance in the pathogenesis of COPD has been postulated (1–3). Oxidative stress enhances inflammation, inactivates protease inhibitors, and directly induces injury to lung epithelial cells. Hence, critical host factors that protect the lungs against oxidative stress may determine the susceptibility to CS-induced lung injury.

One mechanism of defense against toxic compounds and oxidative stress induced by CS exposure is the regulation of

CLINICAL RELEVANCE

Redox imbalance has been implicated in the pathogenesis of chronic obstructive pulmonary disease. Our data suggest that Clara cells are an important source of antioxidant/detoxification gene expression in response to cigarette smoke exposure via the Nrf2 transcription factor.

detoxification and antioxidant genes through antioxidant-responsive elements (ARE). NF-E2-related factor 2 (Nrf2), a member of the “cap ‘n collar” basic leucine zipper transcription factor family, binds to the ARE as a heterodimer with small Maf protein to regulate the expression of several antioxidant and detoxification genes (4, 5). Mice with targeted disruption of Nrf2 have suppressed expression of several ARE-containing antioxidant/detoxification genes (6, 7), making them more susceptible to hyperoxia (8) and CS-induced emphysema (7, 9).

Terminal bronchioles are a key target of CS (10) and are an important site of pathology and pathophysiology in CS-induced lung diseases (11). These airways are the site of increased airway resistance in COPD, which is secondary to structural changes (12), including mucus metaplasia, smooth muscle hyperplasia, and loss of attachments to the surrounding alveolar parenchyma. Although the structure and physiology of the small airways of human smokers have been examined (13–16), little is known about the molecular changes that occur in cells lining terminal bronchioles in response to CS.

CS exposure in mice is widely used to study the effects of CS on the lungs. The model has been exploited mainly for studies of emphysema (17, 18). Small airways have received limited investigation. Accordingly, we have examined the effects of CS exposure on the morphology and gene expression of the cells in the mouse distal airway epithelial cells. We hypothesized that CS exposure would cause changes in the mouse distal airway epithelial cells similar to those in human smokers, in whom goblet cell metaplasia and a loss of Clara cells in the terminal airways are seen (14–16). In addition, we hypothesized that CS would cause changes in the gene expression of the epithelial cells in terminal bronchioles, suggestive of a concerted response to oxidant stress and toxic aromatic hydrocarbons. We have identified several antioxidant and detoxification genes that are expressed by terminal bronchiolar epithelial cells in response to CS exposure. Furthermore, we observed that CS-induced expression of antioxidant and detoxification genes by Clara cells specifically were Nrf2-regulated.

MATERIALS AND METHODS

Smoking Mice

Ten-week-old female C57BL/6 mice obtained from Taconic Farms (Germantown, NY) were housed in a pathogen-free animal facility

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under the veterinary care of the Department of Comparative Medicine at Washington University School of Medicine. Mice were placed in individual smoking chambers ($3 \times 3 \times 9$ cm) and exposed to the smoke of two University of Kentucky 2R1 research cigarettes in a 15-minute period using a 20-ml syringe-driven apparatus, 6 days a week, for up to 6 months. Six months of CS exposure using this system results in significant airspace enlargement (19). Mice were killed 24 hours after the last CS exposure. Age-matched mice were exposed to filtered air and used as controls. All procedures were approved by the Washington University School of Medicine Animal Studies Committee and were performed in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals. Ten independent experiments were performed using at least five mice per condition.

Scanning Electron Microscopy

Sample preparation was performed as previously described (20). Briefly, the lungs were inflation-fixed using Karnovsky's fixative, and the main branching airways to the level of the terminal bronchioles of the right middle lobe were exposed using a dissecting microscope (21). Using a Hitachi S-450 scanning electron microscope, a 100×120 - μ m field adjacent to the bronchoalveolar junction was captured from five terminal airways per mouse using five mice per condition. All cells in each field were examined, and the cell type was determined by morphology. Any cell with cilia present was defined as a ciliated cell; any cell with a central cytoplasmic protrusion was defined as a Clara cell; and any cell with neither characteristic was defined as other. Cell types were expressed as an average number of each cell type per terminal airway $\pm$ SEM.

Staining for Cell-Specific Markers

Sample preparation was performed as previously described (20). Briefly, the lungs were inflation-fixed, paraffin-embedded, and cut into 5- μ m sections. Sections were immunostained for Clara cells (anti-CC10, 1:5,000; kindly provided by Gurmukh Singh, University of Pittsburgh, VA Hospital) or ciliated cells (anti- β -tubulin IV, 30 μ g/ml; Biogenex, San Ramon, CA). Immunostaining was developed using a goat ABC Elite kit or a MOM detection kit (Vector Laboratories, Burlingame, CA), respectively, with a nickel containing DAB substrate and counterstained with nuclear fast red. Mucus cells were detected by periodic acid-Schiff (PAS) staining. Ten terminal airways per mouse from at least five mice per condition were evaluated. The number of positive staining cells per 200 μ m of basement membrane extending proximally from the bronchoalveolar junction was determined. Results are expressed as average number of cells $\pm$ SEM for each condition.

Laser Capture Microdissection

Tissue sample preparation and laser capture microdissection (LCM) were performed as previously described (22). Briefly, lungs were inflated with diluted Tissue-Tek OCT (50% vol/vol OCT in RNase-free 10% sucrose), and 7- μ m frozen sections were ethanol-fixed and stained with Mayer's hematoxylin. Cells in the terminal airway (within 200 μ m of the bronchoalveolar junction) were LCM-retrieved using the PixCell II LCM System (Arcturus Engineering, Mountain View, CA). Approximately 10,000 laser pulses were used to collect the cells from each mouse, and LCM-retrieved cells from at least five mice were pooled for RNA isolation.

RNA Purification, Amplification, and Target Synthesis

Total RNA was extracted from LCM-retrieved cells using a High Pure RNA Isolation Kit (Boehringer Mannheim, Indianapolis, IN) as previously described (22), and the quality and concentration of the RNA was determined using a RNA LabChip (Agilent, Santa Clara, CA) according to the manufacturer's recommendations. The amount of RNA that can be obtained from LCM-retrieved cells is not enough for microarray analysis with a single round of amplification (23). Therefore, 20 ng of RNA was subjected to two rounds of linear amplification before synthesis of the biotin-labeled cRNAs using the procedure described by Luzzi and coworkers (23). Twenty-five micrograms of each biotinylated cRNA target was fragmented and then hybridized to identical Affymetrix Murine Genome Mu74Av2 GeneChips (MG-U74Av2) in the Siteman Cancer Center GeneChip Facility (Washington University School of Medicine, St. Louis, MO).

Analysis of Microarray Data

The images from the scanned microarrays were processed using Affymetrix Microarray Analysis Suite 4.0 and the Average Difference Call, Log Average Ratio, and Absolute Call data from each microarray were exported as text files to be used for numerical analysis using GeneSpring 7.2 software (Silicon Genetics, Redwood City, CA). Normalization and generation of gene lists were performed in a manner similar to that of a previous study (24). Briefly, data were normalized to the 50th percentile of all measurements and to the nonsmoked control sample. Genes that were scored as "absent" by the Affymetrix detection criterion in all samples were excluded. A list of genes that increased expression at least 2.5-fold relative to the nonsmoked control sample was generated. Noting that several of these genes were antioxidants or detoxification genes, we generated a list of antioxidant or detoxification genes and compared this list to a compiled list of known Nrf2-regulated genes (25–29). The complete set of data can be downloaded at <http://bioinformatics.wustl.edu>.

Real-Time RT-PCR

One microgram of total RNA extracted from LCM-retrieved cells was reverse transcribed using random hexamers and the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA) and the resulting products were used as templates for real-time RT-PCR using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) as previously described (30). Assay-on-Demand gene expression probes are shown in Table 1. Relative amounts of each mRNA in the samples were assessed by interpolation of their cycle thresholds from a standard curve and were then normalized against the β_2 -macroglobulin (β_2 -MG) housekeeping gene. Data are representative of at least three independent experiments.

Immunohistochemistry for Nrf2

Sections of lungs of mice exposed to CS for various times were antigen unmasked in 50 mM Tris, pH 8.0 for 5 minutes in a decloaking chamber (Biocare Medical, Walnut Creek, CA) and immunostained for Nrf2 using a rabbit polyclonal anti-Nrf2 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). Immunostaining was detected using a rabbit ABC Elite kit (Vector Laboratories) and a nickel-containing DAB substrate and counterstained with nuclear fast red.

C22 Clara Cells

The immortalized mouse Clara cell line (C22) was generated as previously described (31) from primary Clara cells isolated from the Immortomouse containing a temperature-sensitive, MHC-driven large-T antigen transgene (32). C22 cells were maintained in permissive conditions (33°C in proliferative media containing interferon γ). Before cell treatment, cells were transferred to nonpermissive conditions (39°C in differentiation media without IFN- γ) for 24 hours to inactivate the large-T antigen. Cells were washed twice with serum-free differentiation media before exposure to CS extract.

TABLE 1. ASSAY-ON-DEMAND GENE EXPRESSION PROBES

Name	Assay ID
NF-E2-related factor 2 (Nrf2)	Mm00477784_m1
Nuclear factor kappa B 1 (NFkB1)	Mm00476363_ml
Catalase (Cat)	Mm00437992_m1
Glutamate-cysteine ligase, catalytic subunit (Gclc)	Mm00802655_m1
Glutamate-cysteine ligase, modifier subunit (Gclm)	Mm00514996_m1
Glucose-6-phosphate dehydrogenase (G6pdh)	Mm00656735_g1
Glutathione reductase 1 (Gsr1)	Mm00833903_m1
Heme oxygenase 1 (HO-1)	Mm00516004_m1
Thioredoxin reductase 1 (Txnrd1)	Mm00497442_m1
Cytochrome P450, benz(a)anthracene inducible (Cyp1b1)	Mm00487229_m1
Glutathione-S-transferase, alpha (Gsta1)	Mm00494803_m1
Glutathione S-transferase, mu 2 (Gstm2)	Mm01199654_gH
Glutathione S-transferase omega 1 (Gsto1)	Mm00599866_m1
NADPH: quinone reductase 1 (NQO1)	Mm00500821_m1
Superoxide dismutase 1 (SOD-1)	Mm01344231_g1
UDP-glucose dehydrogenase (Ugdh)	Mm00447643_m1
β_2 -microglobulin (β_2 -MG)	Mm00437764_m1

Preparation of CS Extract

CS extract was prepared using the same syringe-driven apparatus used to expose mice to CS, but the smoke of the two filterless cigarettes was bubbled through 15 ml serum-free media instead of entering the smoking chamber. The resulting suspension (100% CS extract) was then filtered through a 0.22- μ m filter to remove bacteria and large particles. Subsequently, the media were diluted to 5%, 10%, or 20% in serum-free media before incubation with cells. After incubation, cells were harvested for RNA isolation or protein extraction.

Immunofluorescence for 8-Hydroxyguanosine and Nrf2

C22 cells grown in differentiation conditions on tissue culture-treated glass chamber slides (BD Falcon, Bedford, MA) were exposed to CS extract for various times as described above. The cells were washed with PBS and fixed with ice-cold methanol for 20 minutes. The cells were then probed with either a monoclonal anti-8-hydroxyguanosine (anti-8-OHdG) antibody (1:200; QED Bioscience, San Diego, CA) or anti-Nrf2 antibody (1:200; Santa Cruz Biotechnology), followed by FITC-conjugated donkey anti-mouse or anti-rabbit secondary antibody (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. Slides were then mounted with Vectashield mounting media with DAPI (Vector Laboratories).

Antioxidant Detection

C22 cells grown in differentiation conditions in 60-mm plates were exposed to serum-free media alone or containing various percentages of CS extract for 24 hours. The cells were washed twice with cold PBS, scraped into 0.5 ml cold PBS, lysed by sonication, and then centrifuged. An aliquot of each supernatant was used to assess total protein concentration using the DC Protein Assay kit (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations. Equal amounts of each sample were used to determine the total antioxidant potential using the BIOYTECH AOP-490 kit (OXIS International, Portland, OR) according to the manufacturer's instructions. Data are representative of at least three independent experiments performed in triplicate $\pm$ SEM.

Preparation of Whole Cell and Nuclear Extracts

Whole cell extracts were prepared by lysing the C22 cells in 1 ml RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 1 mM PMSF, incubating on ice for 10 minutes, and sonicated. Nuclear extracts were prepared by lysing the cells in 1 ml lysis buffer (20 mM HEPES, pH 7.9, 10 mM KCl, 2 mM MgCl₂, 2 mM EDTA, and 2 mM EGTA) containing protease inhibitor cocktail and PMSF, and incubating on ice for 10 minutes. After microcentrifugation, the supernatant was kept as the cytoplasmic fraction. The pellet was resuspended in 0.5 ml nuclear extraction buffer (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA) containing protease inhibitor cocktail and PMSF, and sonicated. An aliquot of each extract was used to assess protein concentration using the DC Protein Assay kit (Bio-Rad) according to the manufacturer's recommendations. Whole cell extracts, cytoplasmic fractions, and nuclear extracts were stored at -80°C .

Immunoblotting Analysis

Based on the protein concentration, equal amounts of protein of whole cell and nuclear extracts were separated under reducing conditions on an 8% SDS polyacrylamide gel and transferred onto Immobilon-P PVDF transfer membranes (Millipore Corporation, Bedford, MA). After blocking overnight with 5% nonfat dried milk in TBS containing 0.1% Tween 20 at 4°C , the membranes were incubated for 2 hours with anti-Nrf2 (1:2,000; Santa Cruz) antibody, followed by 1 hour of incubation with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1:20,000; Jackson ImmunoResearch). After washing, immunoreactive proteins were detected by chemiluminescence using the ECL Plus Western Blotting Detection System (Amersham Pharmacia, Piscataway, NJ) and subsequent autoradiography.

Inhibition of Nrf2 and NF- κ B by siRNA

Double-stranded siRNA duplexes targeting the expression of the Nrf2 or NF- κ B transcription factors were designed and obtained from Sigma-

Aldrich. Preliminary experiments determined which siRNA duplex to use in subsequent experiments (three siRNA duplexes per gene of interest were evaluated) and the time point for measuring the effects of siRNA (data not shown). A scrambled siRNA duplex was used as a negative control. C22 cells were plated at a density of 5×10^4 cells per well of a 6-well plate. Cells were transfected with 20 nM siRNA duplex using INTERFERin siRNA transfection reagent (Polyplus-Transfection Inc., San Marcos, CA) according to the manufacturer's recommendations. Two days after transfection, cells were exposed to serum-free media alone or containing 10% CS extract for 3 hours, RNA was isolated, and the expression of various antioxidant and detoxification genes were examined by real-time RT-PCR. Results are expressed as fold change relative to untreated conditions. Data are representative of at least three independent experiments $\pm$ SEM.

Assessment of Cell Viability

C22 cells were transfected with 20 nM Nrf2, NF- κ B, or scrambled siRNA duplexes as described above. Two days after transfection, cells were exposed to serum-free media alone or containing various concentrations of CS extract for 24 hours. Detached cells were harvested from the cell culture media by centrifugation, stained with Trypan blue, and counted. The cell-free medium was assayed in triplicate for lactate dehydrogenase (LDH) activity using the TOX-7 *In Vitro* Toxicology Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. Results are expressed as fold change relative to scrambled siRNA-transfected, serum-free media alone conditions. Data are representative of at least three independent experiments $\pm$ SEM.

Statistical Analysis

All analysis was performed with the SPSS 13 program (SPSS, Chicago, IL). Paired Student's *t* test was used to analyze the relationship between untreated and treated conditions. A *P* value of less than 0.05 was considered significant. Data are representative of at least three independent experiments performed in triplicate.

RESULTS

CS Does Not Alter Cell Phenotype in the Terminal Bronchiolar Region

Although the terminal airway epithelium of human smokers has mucus metaplasia and loss of Clara cells (14–16), the effects of CS on the terminal bronchiolar epithelium of mice have received limited investigation. To examine the effects of CS on epithelial cells lining the terminal bronchioles, C57BL/6 mice were exposed to CS for up to 6 months, the lungs harvested, and the airways examined by scanning electron microscopy and immunohistochemistry. Scanning electron microscopy revealed numerous dome-shaped Clara cells interdigitated with ciliated cells in the terminal airways of nonsmoked controls (Figure 1A). The airway epithelium of mice subjected to chronic (6 mo) CS exposure had a more flattened appearance (Figure 1B). The flattening of the Clara cells was not detected at earlier time points (1–28 d) (data not shown). The flattening of the Clara cells induced by chronic CS exposure made the presence of ciliated cells more visible, but the cilia themselves appeared normal (Figure 1D) when compared with the cilia in the terminal bronchioles of nonsmoked controls (Figure 1C). Despite these alterations in physical appearance, morphometric analysis of the terminal bronchioles showed that the average number of Clara and ciliated cells per terminal airway remained the same irrespective of CS exposure (Figure 2A).

Immunostaining analyses confirmed the morphometric analyses performed by scanning electron microscopy. The average number of Clara and ciliated cells in the terminal airway remained unchanged in response to CS exposure (Figure 2B). No goblet cells were detected in the epithelium of the terminal bronchioles of any mice examined by either morphometric appearance or PAS staining (data not shown). These data were further supported by real-time RT-PCR using RNA isolated from LCM-captured

Figure 1. Chronic cigarette smoke (CS) exposure induces flattening of Clara cells in terminal bronchioles. Scanning electron microscopy of lungs harvested from C57BL/6 mice exposed to room air (A and C) or CS (B and D) for 6 months. Higher magnifications are shown in C and D. Images are representative of five terminal airways per mouse from five mice per condition.

terminal bronchiolar epithelial cells, which showed no significant change in gene expression of CCSP (Clara cell–specific protein), *Foxj1* (ciliated cells), or *Muc5ac* (mucus producing cells) in response to chronic CS exposure (data not shown). Together, these data indicate that neither the cell type nor total number of cells changed in the terminal airway of mice in response to CS exposure. This makes the terminal airway gene profile in the mouse of greater interest because the changes represent gene expression alterations of predominately one cell type (Clara cells) and not merely expression patterns due to an alteration in cell type.

Effects of Chronic CS Exposure on Antioxidant and Detoxification Gene Expression in Terminal Bronchiolar Epithelium

Although CS exposure did not alter the epithelial cell profile in the distal airways of mice, CS exposure induced changes in gene expression by terminal bronchiolar epithelium. RNA was isolated from LCM-retrieved terminal bronchiolar epithelial cells of mice that had been exposed to filtered air or CS for 6 months. This regimen of CS exposure resulted in emphysematous-appearing lungs (data not shown). To overcome the biologic variability in individual mice, RNA samples from five mice per condition were pooled for microarray analysis, and two independent experiments (CS exposure, LCM, RNA purification/amplification, and microarray analysis) were performed using different sets of mice. Genes whose expression levels increased at least 2.5-fold in response to CS exposure in both independent experiments were sorted into functional categories using the Affymetrix annotated database for the Mu74Av2 chip. Although this approach may mask other genes that are regulated by CS exposure, only those genes with reproducible changes in expression levels in response to CS exposure in both experiments were reported.

Since the functions of Clara cells are oriented to the protection of the respiratory tract against toxic inhaled substances (33, 34),

Figure 2. CS does not alter cell phenotype in the terminal bronchiolar region. (A) Morphometric and (B) immunohistochemical analyses of the terminal bronchioles of mice exposed to room air (nonsmoking) or CS for 6 months. Ten terminal airways per mouse from five mice per condition were evaluated. Results are expressed as an average number of each cell type per terminal airway $\pm$ SEM.

we focused on the expression of antioxidant and detoxification genes. Of the 486 known genes with at least 2.5-fold increased expression in response to 6 months of CS exposure, 35 were antioxidant or detoxification genes, 21 of which are known to be Nrf2-regulated (25–29), and are shown in bold in Table 2. These include cytochrome P450 1b1 (*CYP1b1*, 18.8-fold), glutathione reductase (*Gsr1*, 4.4-fold), glutamate-cysteine ligase catalytic (*Gclc*, 2.7-fold), and modifier (*Gclm*, 2.5-fold) subunits, members of the glutathione S-transferase (*Gst*) and aldehyde dehydrogenase (*Aldh*) families, as well as Nrf2 itself (3.9-fold). These studies identified several antioxidant/detoxification genes that are expressed by cells in the terminal bronchioles in response to chronic CS exposure.

Chronic CS Exposure Increases Nrf2 Expression in Terminal Bronchiolar Epithelium

To confirm the microarray data, real-time RT-PCR analyses were performed. Terminal bronchiolar epithelial cells constitutively expressed Nrf2 in the absence of CS exposure (Figure 3A). Surprisingly, a significant increase in Nrf2 expression above the levels of the nonsmoked controls was not detected until 6 months of CS exposure. The change in Nrf2 expression level after 6 months of CS exposure determined by real-time RT-PCR (3.2-fold) was similar to that determined by microarray (3.9-fold). These data indicate that only chronic CS exposure up-regulated the expression of Nrf2 in terminal bronchiolar epithelial cells. Additional real-time RT-PCR analyses confirmed the CS-induced expression of several antioxidant and detoxification genes

TABLE 2. EFFECTS OF CHRONIC CIGARETTE SMOKE EXPOSURE ON ANTIOXIDANT AND DETOXIFICATION GENE EXPRESSION IN TERMINAL BRONCHIOLAR EPITHELIUM

Category	Genbank	Description	Ave Fold Change
Antioxidant	AI839138	Thioredoxin interacting protein (Txnip)	5.7
Antioxidant	AW125408	Thioredoxin domain containing 4 (Txnrc4)	4.7
Antioxidant	AI851983	Glutathione reductase 1 (Gsr1)	4.4
Antioxidant	M84147	Alcohol dehydrogenase 5 (Adh5)	3.5
Antioxidant	AI841920	Thioredoxin domain containing 5 (Txnrc5)	3.3
Antioxidant	L39879	Ferritin light chain 1 (Ftl1)	3.2
Antioxidant	M28723	Peroxiredoxin 3 (Prdx3)	3.0
Antioxidant	AB027565	Thioredoxin reductase 1 (Txnrd1)	3.0
Antioxidant	U05809	Transketolase (Tkt)	2.8
Antioxidant	AW046552	Thioredoxin domain containing 14 (Txnrc14)	2.7
Antioxidant	U85414	Glutamate-cysteine ligase, catalytic (Gclc)	2.7
Antioxidant	AF093853	Peroxiredoxin 6 (Prdx6)	2.6
Antioxidant	X52561	Ferritin heavy chain (Fth)	2.6
Antioxidant	X57024	Glutamate dehydrogenase (Gdh)	2.5
Antioxidant	D87896	Glutathione peroxidase 4 (Gpx4)	2.5
Antioxidant	U51167	Isocitrate dehydrogenase 2 (NADP+) (Idh2)	2.5
Antioxidant	U95053	Glutamate-cysteine ligase, modifier (Gclm)	2.5
Detoxification	X78445	Cytochrome P450, 1b1 (Cyp1b1)	18.8
Detoxification	U12961	NAD(P)H dehydrogenase, quinone 1 (Nqo1)	7.5
Detoxification	AW120804	Aldehyde dehydrogenase 9, member A1 (Aldh9a1)	4.1
Detoxification	AF033034	Aldehyde dehydrogenase 3, member A1 (Aldh3a1)	4.0
Detoxification	AI835718	Succinate dehydrogenase, subunit A (Sdha)	3.8
Detoxification	AI839814	Aldo-keto reductase family 1, member A4 (Akr1a4)	3.5
Detoxification	AF061017	UDP-glucose dehydrogenase (Ugdh)	3.2
Detoxification	AI324801	Carbonyl reductase (Cbr3)	2.9
Detoxification	AB025408	Esterase D (EsD)	3.0
Detoxification	M25558	Glycerol-3-phosphate dehydrogenase 1 (Gdc1)	2.8
Detoxification	AB025048	Phosphoribosyl pyrophosphate synthetase 1 (Prps1)	2.8
Detoxification	J04696	Glutathione S-transferase, mu 2 (Gstm2)	2.6
Detoxification	AI837493	NADH dehydrogenase ubiquinone Fe-S 2 (Ndufs2)	2.5
Detoxification	AW123273	Cytochrome P450, 2s1 (Cyp2s1)	2.5
Detoxification	J03928	Phosphofructokinase, liver, B-type (Pfkfb)	2.5
Detoxification	AI843119	Glutathione S-transferase omega 1 (Gsto1)	2.5
Detoxification	AI835461	Aldehyde dehydrogenase 7, member A1 (Aldh7a1)	2.5
Transcription	U70475	p45 NF-E2 related factor 2 (Nfe2l2; Nrf2)	3.9

Bold typeface indicates Nrf2-regulated genes.

detected by microarray, including CYP1b1, Gsr1, Txnrd1, Gclc, Gclm, and Gst family members (data not shown).

Consistent with the real-time RT-PCR data, a low level of Nrf2 protein was detected in the cytoplasm of epithelial cells lining the terminal airway of nonsmoked control mice (Figure 3B). This is consistent with the findings of Cho and colleagues, who localized Nrf2 predominantly in airway epithelium of wild-type mice (6). However, despite no change in Nrf2 expression by 1 d after a single CS exposure (Figure 3A), a dramatic increase in Nrf2 staining in the cells of the terminal bronchioles was detected (Figure 3C). Furthermore, the staining appeared to be more localized to the nucleus as compared with nonsmoked controls. An increase in staining intensity was also detected in the cytoplasm and nucleus of the terminal bronchiolar epithelium after 6 months of CS exposure (Figure 3D). These data suggest that acute CS exposure (1 d) induced the translocation of the Nrf2 transcription factor resulting in Nrf2 protein stabilization, without affecting the Nrf2 expression. In contrast, chronic CS exposure (6 mo) not only induced the activation of Nrf2, but also increased its expression.

CS Directly Induces Antioxidant Activity and Oxidative Stress in Clara Cells

CS contains numerous oxidants (35) that could directly induce oxidative stress on terminal airway epithelial cells. Alternatively, bronchiolar epithelial oxidative stress could be induced by oxidants

produced from inflammatory cells. To determine whether CS directly induces oxidative stress or affects the antioxidant production by Clara cells, we used the C22 cell line derived from primary Clara cells of the Immortomouse (31). C22 cells display many characteristics of mature Clara cells including growth in monolayer, containing dense secretory granules, and production of CCSP and surfactant protein (SP)-A, SP-B, and SP-D, but not SP-C (31). C22 cells were exposed to various concentrations of CS extract for 24 hours, and the total antioxidant potential of the cells was determined by using the AOP-490 assay (36, 37). C22 cells treated with serum-free media alone had measurable antioxidant capacity that was significantly increased after exposure to CS extract (Figure 4A). These data indicate that CS directly induces antioxidant production by Clara cells.

To determine whether CS directly induces oxidative stress in Clara cells, immunostaining for 8-OHdG, a marker of oxidative DNA damage, was performed. 8-OHdG was not detected in C22 cells treated with serum-free media alone (Figure 4B) or after treatment with 5% CS extract (data not shown). When C22 cells were exposed to 10% CS extract, the intensity of 8-OHdG was increased (Figure 4C). However, despite an increase in antioxidant production by cells exposed to 20% CS extract (Figure 4A), condensed nuclei and caspase-3-positive cells were detected (data not shown). At concentrations higher than 20% CS extract, cell rounding and detachment were observed (data not shown). These data suggest that the amount of antioxidant production by C22 cells in response to high concentrations of CS extract was not

Figure 3. Chronic CS exposure increases Nrf2 expression in terminal bronchiolar epithelium. (A) RNAs isolated from laser capture microdissection-retrieved cells from terminal bronchiolar region of mice exposed to CS for various times were subjected to real-time RT-PCR using oligonucleotides specific for murine Nrf2. Data are representative of three separate experiments using two independently isolated RNA samples per time point. Expression of Nrf2 was normalized to that of the β 2-MG housekeeping gene $\pm$ SEM. * $P < 0.005$ relative to nonsmoked control. Sections of lungs from mice exposed to (B) room air, (C) a single CS exposure, or (D) CS repeatedly for 6 months were immunostained using an anti-Nrf2 antibody. (E) Secondary antibody alone served as negative control. Images are representative of four mice per condition.

sufficient for protection against CS-induced apoptosis. Therefore concentrations higher than 10% were not used for subsequent experiments.

CS Induces Clara Cell Expression of Several Antioxidant and Detoxification Genes

To determine which antioxidant genes were up-regulated in C22 cells in response to CS, real-time RT-PCR analyses were performed using RNA isolated from C22 cells exposed to serum-free media alone or containing 10% CS extract for 3 hours. Several antioxidant genes were up-regulated by C22 cells in response to CS (Figure 5A), including heme oxygenase 1 (37.3-fold), glutamate-cysteine ligase catalytic (2.8-fold) and modifier (8.3-fold) subunits, and glutathione reductase (3.6-fold). In addition, several detoxification genes were up-regulated (Figure 5B), including cytochrome P450 1b1 (10.0-fold), NQO1 (9.6-fold), superoxide dismutase (2.5-fold), and members of the glutathione S-transferase family. These data are similar to the microarray data using RNA isolated from LCM-retrieved terminal bronchiolar epithelial cells (Table 2).

Figure 4. CS extract directly induces oxidative stress in C22 cells. (A) C22 cells were exposed to serum-free media alone or containing various amounts of CS extract (CSE) for 24 hours, and the total antioxidant potential of the cells was determined. Data are representative of three independent experiments performed in triplicate $\pm$ SEM. * $P < 0.005$; ** $P < 0.05$ relative to 0% CS extract. C22 cells were exposed to serum-free media alone (B and D) or media containing 10% CS extract (C and E) for 24 hours and stained for oxidative DNA damage using an anti-8-OHdG antibody (B and C). Nuclei were detected with DAPI (D and E).

Exposure of C22 Cells to CS Extract Results in Nrf2 Stabilization

We observed that CS exposure resulted in increased Nrf2 staining in the cells of the terminal bronchioles *in vivo* by 1 day after CS exposure, a time point at which Nrf2 expression was not yet induced (Figure 3). Therefore, we examined whether CS would affect Nrf2 expression and/or protein stabilization in C22 cells. Similar to terminal bronchiolar epithelial cells, untreated C22 cells express basal levels of Nrf2 and treatment of C22 cells with 10% CS extract for up to 24 hours failed to induce Nrf2 expression (Figure 6A). However, treatment of C22 cells with CS extract caused an increase in Nrf2 protein within 1 hour of exposure, which plateaued after 3 hours of exposure, but persisted over the 24-hour period (Figure 6B). These data indicate that the CS-induced increase in the amount of Nrf2 was caused by protein stabilization rather than by transcriptional activation.

A Antioxidant **B Detoxification**

Figure 5. CS extract induces C22 cell expression of several antioxidant and detoxification genes. RNAs isolated from C22 cells exposed to serum-free media alone (open bars) or containing 10% CS extract (solid bars) for 3 hours were subjected to real-time RT-PCR using gene-specific oligonucleotides for several (A) antioxidant and (B) detoxification genes. After normalization to β 2-MG, results are expressed as fold change above untreated conditions. Data are representative of three independent experiments $\pm$ SEM.

Nrf2 Is Translocated to the Nucleus of C22 Cells in Response to CS

The translocation of Nrf2 from the cytoplasm to the nucleus has been shown to be essential for the induction of several antioxidant and detoxification genes. To determine whether CS induces Nrf2 translocation, we examined the localization pattern of Nrf2 in untreated and CS extract-treated C22 cells by immunofluorescence. In the absence of CS extract, Nrf2 was detected in the cytoplasm of C22 cells, but was detected in the nucleus of C22 cells within 1 hour of exposure to 10% CS extract (Figure 6C). Western blot analysis of nuclear extracts using the Nrf2 antibody confirmed that Nrf2 protein accumulated rapidly (as early as 30 min) in the nucleus after exposure to CS extract (Figure 6D). These data show that CS induced the translocation of Nrf2 to the nucleus of C22 cells. In addition, we found that CS also induced nuclear translocation of NF- κ B in C22 cells (data not shown). This is consistent with the findings of Kode and coworkers, who

Figure 6. Exposure of C22 cells to CS extract results in Nrf2 translocation to the nucleus and stabilization, but does not increase Nrf2 expression. (A) RNA isolated from C22 cells exposed to serum-free media alone or containing 10% CS extract for up to 24 hours were subjected to real-time RT-PCR using Nrf2-specific oligonucleotides. After normalization to β 2-MG, results are expressed as fold change above untreated conditions. Data are representative of three independent experiments $\pm$ SEM. (B) Western blot analysis of whole cell protein extracts collected from C22 cells exposed to CS for up to 24 hours using an Nrf2-specific antibody. The same membrane was also probed with a GAPDH-specific antibody to control for loading. Data are representative of at least three independent experiments. (C) C22 cells were exposed to serum-free media containing 10% CS extract for up to 1 hour. Cells were fixed and stained using a Nrf2-specific antibody. (D) C22 cells were exposed to serum-free media containing 10% CS extract for up to 1 hour and the amount of Nrf2 located in the cytoplasmic (C) and nuclear (N) fractions was determined by Western blot analysis using an Nrf2-specific antibody. Data are representative of at least three independent experiments.

found that CS induced NF- κ B translocation in human small airway epithelial cells (38).

CS-Induced Antioxidant and Detoxification Gene Expression Are Regulated by Nrf2 and NF- κ B Transcription Factors

To determine whether CS induces antioxidant and detoxification gene expression via Nrf2 activation, and whether Nrf2 activation

plays a role in protection against CS-induced oxidative damage, we examined the effects of Nrf2 siRNA on gene expression and cell viability after CS exposure. In addition, since CS induces the translocation of NF- κ B, which is a regulator of expression of antioxidant genes in response to oxidative stress (39, 40), we examined whether CS-induced antioxidant and detoxification gene expression was NF- κ B mediated. First, to confirm that the Nrf2 and NF- κ B siRNA duplexes effectively and selectively inhibited the expression of Nrf2 and NF- κ B, respectively, the siRNA duplexes were separately transfected into C22 cells, and 48 hours after transfection, RNA was isolated and the amount of Nrf2 and NF- κ B message was quantified by real-time RT-PCR. C22 cells transfected with Nrf2 or NF- κ B siRNA duplexes showed significant reductions in Nrf2 (78%) or NF- κ B (76%) messages, respectively (Figure 7A). C22 cells were separately transfected with scrambled siRNA duplex as a negative control. Transfection of a scrambled siRNA duplex did not affect the expression of these genes. Furthermore, transfection of these

siRNA duplexes did not alter the expression of the housekeeping gene β 2-MG (data not shown), indicating that the siRNA duplexes did not affect overall gene expression.

To determine the impact of Nrf2 and NF- κ B transcription factor activation on the protection against CS-induced cell death, C22 cells were transfected with siRNA duplexes for either Nrf2 or NF- κ B. Two days after transfection, cells were exposed to serum-free media alone or containing various concentrations of CS extract for 24 hours, and the level of LDH activity, an indicator of cell death, in the culture media was measured. C22 cells transfected with the scrambled siRNA had slightly increased LDH activity in the culture media when exposed to 10% CS extract as compared with the cells that were exposed to 0% CS extract (Figure 7B). In contrast, cells with decreased Nrf2 expression by siRNA were dramatically more susceptible to CS exposure, as indicated by a significant increase in LDH activity in the conditioned media, even at a concentration of 2.5% CS extract. C22 cells with diminished NF- κ B expression also released sig-

Figure 7. CS-induced antioxidant and detoxification gene expression is primarily regulated by the Nrf2 transcription factor. (A) C22 cells were transfected with siRNA duplexes targeting the expression of the Nrf2 or NF- κ B transcription factors, or a scrambled siRNA duplex. Two days after transfection, RNAs were isolated and subjected to real-time RT-PCR for expression of Nrf2 or NF- κ B, respectively. Data are representative of at least three independent experiments $\pm$ SEM. * P < 0.005 relative to nontransfected control. (B) In separate experiments, 2 days after transfection with scrambled (Scr, open squares), Nrf2 (solid squares), or NF- κ B (shaded squares) siRNA duplexes, C22 cells were exposed to serum-free media containing 0–10% CS extract for 24 hours and the relative amount of lactate dehydrogenase (LDH) in the media was determined. Results are expressed as fold change relative to C22 cells that were transfected with the Scr siRNA, exposed to serum-free media alone. (C and D) Two days after transfection with Scr (open bars), Nrf2 (solid bars), or NF- κ B (shaded bars) siRNA duplexes, C22 cells were exposed to serum-free media containing 10% CS extract for 3 hours. RNAs were isolated and subjected to real-time RT-PCR using gene-specific oligonucleotides for several (C) antioxidant and (D) detoxification genes. After normalization to β 2-MG, results are expressed as fold change relative to C22 cells transfected with the Scr siRNA. Data are representative of at least three independent experiments $\pm$ SEM. * P < 0.005; ** P < 0.05 relative to scramble siRNA control.

nificantly more LDH, but only after exposure to higher concentrations of CS extract. Furthermore, exposure of C22 cells that were transfected with the scrambled siRNA to 10% CS extract resulted in approximately 0.05% cell detachment, whereas exposure of C22 cells transfected with the Nrf2 or NF- κ B siRNA to 10% CS extract caused approximately 20% and 14% cell detachment, respectively (data not shown). These data suggest that both Nrf2 and NF- κ B play a role in the protection against CS-induced cell death, possibly via induction of antioxidant and detoxification genes.

To determine which, if any, of the CS-induced antioxidant and detoxification genes were mediated through Nrf2 or NF- κ B activation, C22 cells were transfected with siRNA duplexes for either Nrf2 or NF- κ B, and 2 days after transfection, cells were exposed to serum-free media alone or containing 10% CS extract for 3 hours, RNA was isolated, and the expression of various antioxidant and detoxification genes were examined by real-time RT-PCR. Decreases in Nrf2 expression by siRNA resulted in corresponding decreases in the CS-induced expression of every antioxidant gene examined with the exception of Txnrd1 (Figure 7C), while affecting the expression of few detoxification genes, namely Gsta1 and NQO1 (Figure 7D). However, reduction of NF- κ B by siRNA only slightly inhibited the CS-induced expression of catalase and Gstm2. Thus, Nrf2 is a prominent regulator of CS-induced antioxidant genes by C22 cells.

DISCUSSION

COPD represents the clinical expression of complex alterations in structure and function of alveolar tissue and small airways. The structural changes are emphysema and bronchiolar inflammation and remodeling. The mouse model for CS-induced lung injury is widely used to study emphysema (17, 18); however, the small airways in this model have received limited investigation. We hypothesized that CS exposure would cause changes in the epithelial cell phenotype in the distal airways of mice, since human smokers demonstrate a loss of Clara cells and develop goblet cell metaplasia in the terminal airways (14–16). However, morphometric and immunohistochemical analyses detected no change in cell type or number per terminal bronchiole and only a slight morphologic alteration, with the Clara cells showing a flattened appearance. We speculate that the flattening of the Clara cells is due to shedding of the apical cap during secretion (41), potentially of antioxidants or detoxifying enzymes. Flattened, nonciliated cells have been detected by scanning electron microscopy in human smokers (15); however, many of them had microvilli and therefore were considered to be goblet cells rather than flattened Clara cells. We were unable to detect the presence of goblet cells in the terminal bronchiole of mice after CS exposure. Our studies used C57BL/6 mice, a strain that develops airspace enlargement from chronic CS exposure (18). The effects of CS exposure are strain-dependent with respect to emphysema development (17, 18); therefore, the use of more sensitive strains might show phenotypic changes typical of humans with COPD. Consistent with this, March and colleagues found that CS exposure induced mucus cell hyperplasia in the epithelium in the large airways of A/J mice (42). However, similar to our findings, remodeling of the small airways, a key contributor to the airway resistance in humans with COPD, was not detected in the CS-exposed A/J mice.

LCM and Microarray

Gene expression profiling of whole lung samples reflects heterogeneity of cellular components and has the disadvantage of averaging-out signals, such that low-level signals from small, but potentially critical, cell populations could go underdetected.

To identify genes that were differentially expressed in CS-exposed airway epithelium in mice, we used LCM to selectively isolate terminal airway epithelial cells. However, LCM presents a challenge in retrieving sufficient quantities of RNA for microarray analysis, resulting in the need for amplification (23, 43). Although there is a 3' bias in the amplification process, the reproducibility and usefulness of RNA amplification in gene expression profiling has been reported (23, 44). The abundant expression of Clara cell-specific genes in our LCM-derived sample compared with whole lung analysis (data not shown) confirms the selectivity of our method, and the gene expression profile from two independent experiments involving RNA amplification was highly correlated. Further evidence that the method of RNA amplification still reflected absolute gene expression were the similarities between changes in gene expression detected by microarray, which used amplified RNA, and real-time RT-PCR, which used unamplified RNA. For example, the change in Nrf2 expression level after 6 months of CS exposure determined by real-time RT-PCR (Figure 3A) and microarray (Table 2) were 3.2-fold and 3.9-fold, respectively. Therefore, a combination of LCM and microarray to analyze cell-specific gene expression could provide a screening tool to identify and validate the altered expression of novel genes in the terminal airway epithelium after CS exposure.

During the process of LCM sampling, all cells lining the bronchoalveolar junction were captured. Although the sample was highly enriched in Clara cells, we did not exclude other cell types, such as ciliated cells or infiltrating inflammatory cells. Therefore, we cannot rule out the possibility that the changes in gene expression detected in the LCM-captured cells were in part due to large changes in gene expression by cells less abundant than Clara cells. However, C22 cells showed great similarity with our *in vivo* studies, indicating that CS induces expression of several antioxidants and detoxification genes and that acute CS exposure promotes Nrf2 protein stability rather than inducing its expression. Therefore, the Clara cell is an important source of antioxidant/detoxification gene expression in response to CS exposure by airway epithelial cells.

Small Airways in COPD versus Mice Exposed to CS

The correlation of small airway narrowing, whether it be from infiltrating inflammatory cells, airway remodeling, or untethering of alveolar attachments, and physiologic impairments suggests a direct contribution of the small airways to the functional deterioration seen in COPD (45, 46). Studies have detected increased levels of oxidant markers in biological samples from patients with COPD, and oxidative stress has been associated with airway narrowing (47, 48). Although our evaluation demonstrates that mice do not develop overt structural changes of the small airways, our data suggest that the mouse CS exposure model is valid for identification of antioxidant and detoxifying enzymes produced in response to CS, and determination of factors involved in the regulation of the oxidant/antioxidant balance. In fact, despite the lack of terminal airway goblet cell metaplasia in the mouse, the gene profile, particularly in regard to antioxidant and detoxification genes, was remarkably similar to published results of human small and/or large airway epithelial cells (49, 50). Therefore, antioxidant defense mechanisms in the small airways may have an important role in the susceptibility to CS-induced lung injury.

Glutathione Redox System in Response to CS Exposure

Our data suggests a coordinated response of the glutathione system to CS exposure. Reduced glutathione (GSH) provides protection to the lung from oxidative injury by acting as a supply

for reduced thiol equivalents. GSH is important in intracellular antioxidant reactions, but is also secreted into the lung surface liquid (51). In fact, GSH levels in the lung surface liquid are 100 times greater than levels in plasma. GSH is supplied by *de novo* synthesis and recycling of oxidized glutathione (GSSG). Gclc and Gclm together form glutamylcysteine ligase (formerly γ -glutamylcysteine synthetase), the enzyme responsible for the rate-limiting step in GSH synthesis. Glutathione reductase (Gsr1) catalyzes the reduction of GSSG to GSH using NADPH. Transketolase (Tkt) is an important enzyme in the supply of NADPH via the pentose phosphate pathway. Several of these genes were up-regulated in response to CS exposure by terminal bronchiolar epithelial cells (Table 2), including Gclc, Gclm, Gsr1, and Tkt. In addition, our *in vitro* data confirms the Nrf2-dependence of glutathione supply in Clara cells (Figure 5). This result suggests that alterations in the glutathione supply may be important in the susceptibility of Nrf2 KO mice to CS exposure and/or COPD in humans.

In addition to the up-regulation of genes involved in the production of GSH, our profiling data suggested that enzymes that use glutathione for detoxification and peroxidation were also up-regulated in the airway epithelium in response to CS exposure. Glutathione S-transferases (Gst1, Gstm2) and a glutathione peroxidase (Gpx)-4 were all up-regulated in mice after 6 months of CS exposure (Table 2). Gst and Gpx family members are commonly up-regulated in the airway epithelium of human smokers as well (49, 50, 52). However, the up-regulation of Gpx4 in our samples is interesting because Gpx2 and not Gpx4 was up-regulated in mouse whole lung and human epithelial cell profiling experiments (49, 50, 52). Its up-regulation may be an example where the enrichment of Clara cells of our LCM-derived sample unmasked a novel gene that otherwise was not identified in a profile of mixed lung cells.

Detoxification Genes

The enzymes of the cytochrome P450 superfamily are the major enzymes implicated in the metabolism of both xenobiotics and endobiotics (53). Many of the carcinogens in CS, such as polycyclic aromatic hydrocarbons and benzo(a)pyrene, are catalyzed by CYP1a1 and CYP1b1. CYP1b1 is one of the most highly up-regulated detoxification genes after CS exposure. However, our data suggest that much of the increase in CYP1b1 expression seen in Clara cells is independent of Nrf2. Although we cannot rule out an effect due to incomplete suppression of Nrf2 (only 76% suppression by siRNA), our finding is consistent with primary regulation of CYP1b1 synthesis by the aromatic hydrocarbon receptor (Ahr), a ligand-activated transcription factor, which binds to a xenobiotic response element (XRE).

In addition to the phase I detoxifying genes (like CYP450 family members), aldehyde dehydrogenase (Aldh) and aldo-keto reductase (Akr) families are represented in the genes up-regulated at 6 months (Table 2). Among these genes Aldh3 was also up-regulated in small and large airway samples of human smokers (49, 50, 52). Aldh members catalyze the detoxification of acrolein, which is known to be present in CS, and aldehydes that are formed by alcohol dehydrogenases (note Adh5 was also up-regulated). Although the Akr1a4 family member that was up-regulated in our gene profile is present only in the mouse, human airway samples also demonstrate up-regulation of the Akr1 family members (49, 50, 52). Akr family members are involved in the reduction of aromatic aldehydes that are present in CS, but there may be redundancy in function with the previously mentioned Gst family. The importance of individual family members and the differences between mice and humans or even strains of mice (CS resistant versus sensitive) could be an important area of future research for both COPD and lung cancer prevention.

CS Results in Nrf2 Activation and Stabilization

The transcription factor Nrf2 plays a central role in the cellular defense against oxidative stress by orchestrating the expression of several antioxidant and detoxification genes (4, 5). Nrf2 is constitutively expressed in various tissues and, under normal conditions, resides in the cytoplasm bound to its negative regulator Keap1 (Kelch-like ECH-associating protein 1), becomes ubiquitinated, and degraded in proteosomes. Activation of Nrf2 is mediated by mechanisms that cause its dissociation from Keap1 and promote its translocation into the nucleus to drive gene expression. Therefore, Nrf2 activity can be prolonged by mechanisms that affect the rate of Nrf2 degradation, which results in its stabilization (4, 5, 54, 55). However, recent data show that increasing Nrf2 protein translation rather than protein stability also can enhance Nrf2 activity (56).

By immunohistochemistry, we detected an increase in Nrf2 protein in terminal bronchiolar epithelial cells of mice 24 hours after a single CS exposure, while an increase in Nrf2 RNA was not detected until exposure to CS for 6 months (Figure 3). Similarly, *in vitro* studies using the C22 Clara cells revealed that the levels of Nrf2 protein increased in cells after exposure to CS extract, while the level of Nrf2 RNA remained unchanged (Figure 6). Increasing Nrf2 translocation and protein stability would lead to enhanced transcriptional activity, suggesting that the regulation of Nrf2 stability may represent an important mechanism in the activation of antioxidant/detoxification gene expression after acute CS exposure. However, we detected an increase in Nrf2 expression after chronic CS exposure. Therefore, it appears that Nrf2 regulates the expression of antioxidant and detoxification genes in response to CS exposure by more than one mechanism.

CS-Induced Antioxidant/Detoxification Gene Expression Is via Nrf2, not NF- κ B

Studies have identified regulatory elements in the promoter regions of several detoxification and antioxidant genes. The ARE is present in numerous genes and found to be bound by Nrf2 to induce downstream gene expression (4, 5, 57). Although the AP-1 binding site has sequence similarity to the ARE sequence, AP-1 is not involved in ARE-mediated gene expression (5). In addition to Nrf2, NF- κ B regulates the expression of components of the glutathione redox system, such as Gclc, Gclm, and glutathione synthase, in response to oxidative stress (39, 40). However, we found that antioxidant and detoxification gene expression induced by CS exposure is primarily controlled by Nrf2. After exposure to CS, Nrf2 translocates to the nucleus and becomes stabilized, coinciding with increased expression of several antioxidant and detoxification genes. Depletion of Nrf2 levels by siRNA significantly attenuated expression of many CS-induced antioxidant and some detoxification genes, whereas siRNA-induced decreases in NF- κ B did not. Taken together, these results show that Nrf2 has a prominent role in orchestrating the transcriptional response to agents in CS.

Nrf2 and NF- κ B Are Important for Protection Against CS-Induced Cell Death

CS has been shown to induce cell death of alveolar macrophages, lung endothelial cells, and various lung epithelial cell types (38, 58–61). We found that exposure of C22 cells, a Clara cell line, to high concentrations of CS extract ($\geq 20\%$) induces oxidative stress, condensed nuclei, an increase in caspase 3–positive cells, and cell rounding and detachment. The susceptibility of the lung to CS has been shown, at least in part, to be determined by the regulation of protective antioxidant systems. For example, Nrf2-deficient mice have suppressed expression of several antioxidant and detoxification genes (6, 7), and after 4 to 6 months of CS

exposure, these mice exhibited increased inflammation, oxidative damage, and lung cell apoptosis, resulting in enhanced emphysema development (7, 9). However, because these studies involved chronic CS exposure, a direct role for Nrf2 activation in the protection against CS-induced lung damage could not be ascertained. Our studies involving siRNA transfection to diminish Nrf2 expression, albeit simplistic using a single cell type *in vitro*, more directly address this question. We found that C22 cells with depleted Nrf2 showed a decrease in expression of several antioxidant genes and were much more susceptible to CS-induced cell death. Together, these studies emphasize the importance of Nrf2-mediated antioxidant response for protection against CS-induced lung epithelial cell damage. However, at higher doses or duration of CS exposure, the oxidative stress becomes greater than the antioxidant potential, despite an up-regulation of several antioxidants, and CS-induced apoptosis occurs. Cells with decreased NF- κ B were also more susceptible to CS-induced cell death, but to a lesser degree than cells with depleted Nrf2. Since the reduction in NF- κ B expression did not alter the CS-induced expression of the antioxidant and detoxification genes examined, the mechanism by which NF- κ B is protective against CS-induced cell death is likely different than that of Nrf2.

In summary, using a strain of mice susceptible to CS-induced emphysema, we have shown that CS exposure failed to induce morphologic changes in the terminal bronchiolar region of mice. However, CS exposure induced expression of several antioxidant and detoxifying enzymes in a pattern similar to the airway epithelium of human smokers. In addition, we showed that CS exposure increased Nrf2 protein in bronchiolar epithelium with a nuclear localization, suggesting Nrf2 activation. Using *in vitro* studies, we have shown that CS directly induced oxidative stress and the stabilization and nuclear translocation of Nrf2 in Clara-like cells, which correlated with the induction of antioxidant and detoxification genes by these cells. Furthermore, decreasing Nrf2 expression by siRNA resulted in a corresponding decrease in CS-induced expression of several antioxidant and detoxification genes and increased susceptibility to CS-induced cell death, whereas decreasing NF- κ B had less of an effect. Thus, the protective response by the Clara cell to CS exposure is predominantly regulated by Nrf2.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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