Comprehensive gene expression profiling of rat lung reveals distinct acute and chronic responses to cigarette smoke inhalation

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Stevenson CS, Docx C, Webster R, Battram C, Hynx D, Giddings J, Cooper PR, Chakravarty P, Rahman I, Marwick JA, Kirkham PA, Charman C, Richardson DL, Nirmala NR, Whittaker P, Butler K. Comprehensive gene expression profiling of rat lung reveals distinct acute and chronic responses to cigarette smoke inhalation. Am J Physiol Lung Cell Mol Physiol 293: L1183–L1193, 2007. First published August 24, 2007; doi:10.1152/ajplung.00105.2007.—Chronic obstructive pulmonary disease (COPD) is a smoking-related disease that lacks effective therapies due partly to the poor understanding of disease pathogenesis. The aim of this study was to identify molecular pathways that could be responsible for the damaging consequences of smoking. To do this, we employed Gene Set Enrichment Analysis to analyze differences in global gene expression, which we then related to the pathological changes induced by cigarette smoke (CS). Sprague-Dawley rats were exposed to whole body CS for 1 day and for various periods up to 8 mo. Gene Set Enrichment Analysis of microarray data identified that metabolic processes were most significantly increased early in the response to CS. Gene sets involved in stress response and inflammation were also upregulated. CS exposure increased neutrophil chemokines, cytokines, and proteases (MMP-12) linked to the pathogenesis of COPD. After a transient acute response, the CS-exposed rats developed a distinct molecular signature after 2 wk, which was followed by the chronic phase of the response. During this phase, gene sets related to immunity and defense progressively increased and predominated at the later time points in smoke-exposed rats. Chronic CS inhalation recapitulated many of the phenotypic changes observed in COPD patients including oxidative damage to macrophages, a slowly resolving inflammation, epithelial damage, mucus hypersecretion, airway fibrosis, and emphysema. As such, it appears that metabolic pathways are central to dealing with the stress of CS exposure; however, over time, inflammation and stress response gene sets become the most significantly affected in the chronic response to CS.

Chronic obstructive pulmonary disease; metabolic pathways; oxidant stress

CIGARETTE SMOKE (CS) is the main etiologic factor for developing chronic obstructive pulmonary disease (COPD), the fourth most common cause of chronic morbidity and mortality in the world (23). However, not all smokers develop COPD, suggesting that there are additional genetic factors that influence disease susceptibility. The COPD phenotype is typified by an accelerated rate of age-related decline in lung function that is related with structural changes in the lung, including peribroncholar fibrosis, emphysema, and mucus hypersecretion. The disease is defined by the NHLBI/WHO Global Initiative as “a disease state characterized by airflow limitation not fully reversible . . . associated with an abnormal inflammatory response of the lungs to noxious particles and gases” (28). The inflammation is characterized by the infiltration of neutrophils, macrophages, and lymphocytes into the lung tissue and airways that persists after smoking cessation. Unfortunately, much of what is known about the pathogenesis of COPD originates from clinical observations from a single time point that, although they may imply potential mechanisms, cannot distinguish between causative and consequential factors of the disease process. Consequently, little is known about the sequence of events that leads to the resulting lung pathologies or the molecular pathways involved in effecting these changes.

Because of these clinical limitations, animal models have played an important role in attempts to elucidate disease-related mechanisms. A variety of agents have been used to mimic COPD-like changes in the lungs of rodents, including elastase, sulphur dioxide, nitrogen dioxide, ozone, lipopolysaccharide, and coal dust (reviewed in Ref. 21). Although some of these models have been important for understanding of how some of these pathological changes may occur, how these models correlate with the changes that occur in response to cigarette smoking remains unclear. There have also been a number of descriptive studies investigating the acute and chronic effects of CS exposure in rodents (reviewed in Refs. 38 and 39, respectively). Most of these studies were largely focused on characterizing the inflammatory and/or pathologic changes that resulted, whereas only a few have focused on global molecular changes (9). In addition, few studies have characterized the effects of acute and chronic exposures within the same study (7). The use of knockout and transgenic animals in smoking studies has provided important insights into potential molecular mechanisms that are linked to disease pathogenesis (10a, 19, 29). These studies are powerful but limited to demonstrating the protective or destructive effects attributed to the abrogation or gross overexpression of a single gene. Because COPD susceptibility is likely to be heterogeneous and

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polygenic, understanding how macro changes at the level of the gene expression translate into pathological and physiological changes in response to CS will be essential for understanding how the disease develops.

The aim of this study was to identify the molecular changes induced by CS inhalation that may drive the biological and pathological consequences leading to disease. To do this, Sprague-Dawley rats were exposed to CS for 1 day and up to 34 wk. In addition, we studied the effect of smoking cessation by exposing one group for 26 wk followed by 8 wk of no exposures. Using Gene Set Enrichment Analysis, global changes in gene expression were translated into alterations in sets of genes with common molecular functions. We then measured protein levels of some of the genes for which expression changed in response to CS. We also characterized inflammatory cell infiltration, oxidant-induced damage to cells in the lung, pathologies at every level of the airways, and lung function. Here, we demonstrate a clear distinction between the acute and chronic response to CS and provide a new hypothesis to explain why these changes occur, resulting in the pathologies linked to COPD.

MATERIALS AND METHODS

Statement on animal welfare. Studies described herein were performed under a Project License issued by the United Kingdom Home Office, and protocols were approved by the Local Ethical Review Process at the Novartis Institutes for Biomedical Research, Horsham, West Sussex, United Kingdom.

Animal maintenance conditions. Male Sprague-Dawley rats (350–400 g; Charles River, Margate, Kent, United Kingdom) were housed in rooms maintained at constant temperature (21 ± 4°C) and humidity (55% ± 15%) with a 12-h light cycle and 15–20 air changes per hour. Two animals were housed per cage containing two nest packs (55%–60% humidity) with a 12-h light cycle and 15–20 air changes per hour. Two animals were housed per cage containing two nest packs filled with grade 6 sawdust (Datesand, Manchester, United Kingdom), nesting material (Enviro-Dri, Lillico), maxi fun tunnels, and Aspen chew blocks (Lillico) to provide environmental enrichment. Animals were allowed food, RM1 pellets (SDS), and water ad libitum.

CS exposure. Animals were exposed to whole body smoke as previously described (32) with some minor modification. Briefly, animals were exposed to 30 ml of smoke every 60 s with fresh air being pumped in for the remaining time. The smoke was generated using 2R1 research cigarettes (University of Kentucky, Lexington, KY), and sham control animals were exposed to room air only for the same duration of time (60 min per exposure period). Animals were killed with an overdose of terminal anesthetic (sodium pentobarbitone, 200 mg/ip) followed by exsanguinations 2 h after the last smoke exposure period. The carboxyhemoglobin levels were ~5% at the time of termination. After 3 wk of exposures, plasma cotinine levels were assayed using a mucin enzyme-linked lectin assay (ELLA) as previously described (22). Macrophages and neutrophils were stained as previously described (22). Macrophages were stained for ED1, and neutrophils were identified with the substituted naphthol method for AS-D chloroacetate esterase. Cells were identified using an Axiolim home microscope by both positive staining and standard morphological criteria. Staining intensity of 4HNE was assessed in alveolar macrophages semiquantitatively in a blinded fashion and graded 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. A Mann-Whitney’s nonparametric statistical test was performed to determine significance of data.

Preparation of bronchoalveolar lavage fluid and lung tissue. Rats not used for microarray analysis were culled as above, and lungs were lavaged with 3 × 4-m1 aliquots of sterile PBS. All aliquots were combined for individual rats. Bronchoalveolar lavage (BAL) total cell counts and differentials were performed as previously described (32). The right lung lobes were tied off, snap-frozen in liquid nitrogen, and used for protein analysis. The trachea and left lung were inflated with 5 ml of 10% neutral buffered formalin (NBF) for histological analysis.

Immunohistochemistry for 4HNE. Immunohistochemistry for 4-hydroxy-2-nonenal (4HNE) was performed by microwave irradiation method. Briefly, lung sections were dewaxed in xylene, rehydrated, and endogenous peroxidase-inhibited with 0.5% hydrogen peroxide in methanol for 10 min. Sections were stained with anti-4HNE adduct (Calbiochem) overnight at 4°C. Immunodetection was performed using biotinylated rabbit anti-mouse Ig reagent (Dako Cytomation, Cambridgeshire, United Kingdom), SABC reagent (Dako Cytomation, Cambridgeshire, United Kingdom), and diaminobenzidine (Sigma, Dorset, United Kingdom). The nuclei were counterstained with Cole’s hematoxylin solution. Tonsil was used as a positive control, and for negative controls the primary antibody was omitted from one section of each of the samples. Cells were identified by both positive staining and standard morphological criteria. Staining intensity of 4HNE was assessed in alveolar macrophages semiquantitatively in a blinded fashion and graded 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. A Mann-Whitney’s nonparametric statistical test was performed to determine significance of data.

BAL fluid and tissue cytokine analysis. Cell-free BAL and tissue homogenate supernatants were used to measure cytokine levels by ELISA (R&D Systems, Abingdon, United Kingdom, except for GRO/CINC-1, Amersham). Tissue homogenate protein levels were measured using the Bio-Rad protein assay (Bio-Rad, Hertfordshire, United Kingdom), and cytokine values were normalized against protein levels for individual homogenate samples.

Determination of BAL fluid mucin concentrations. Samples were assayed using a mucin enzyme-linked lectin assay (ELLA) as previously described (3, 15).

Immunohistochemical staining of inflammatory cells. Macrophages and neutrophils were stained as previously described (22). Macrophages were stained for ED1, and neutrophils were identified with the substituted naphthol method for AS-D chloroacetate esterase. Cells were identified using an Axiolim home microscope by both positive staining and standard morphological criteria.

Immunohistochemical staining of mucin and quantification of goblet cell density. Both tracheal and primary bronchus mucin were detected by a two-stage immunoperoxidase method using Ulex eur-
*paeus* agglutinin-1 (UEA-1; Sigma) validated by Jackson and colleagues (15) as previously described (32).

**Staining for collagen and smooth muscle.** Sections (3 μm) were incubated in 0.1% Sirius Red F3BA (VWR, Poole, United Kingdom) to identify collagen or with mouse anti-human smooth muscle actin, clone 1A4 (Dako Cytomation, Ely, United Kingdom; see supplementary methods online at the AJP-Lung Cellular and Molecular Physiology web site for further detail). The areas of collagen and smooth muscle actin staining were assessed under a Zeiss Axioplan 2 microscope (×10 magnification) with an Imaging Associates KS400 image analyzer (Imaging Associates, Bicester, United Kingdom). Values were determined by measuring the inside perimeter of the airway and dividing it by the area of pigmented pixels, which was then converted into micrometers to give an average thickness surrounding the airway. To avoid bias on slant cuts, where the airway wall is thicker, a constant pixel count away from the airway was always measured when calculating the area. This prevented scoring of pseudo-area of pigmented tissue owed to more staining on obliquely cut airways. Collagen and smooth muscle actin were quantified on areas of the upper, upper-middle, lower-middle, and lower segments of the lung. The mean thickness for each sample was determined, and then the group (smoke- or air-exposed) was averaged to give a mean and standard error of the mean (SE) for each time point. A Mann-Whitney’s nonparametric statistical test was performed to determine significance of data.

**Quantifying mean linear intercept.** Mean linear intercept was measured manually using a method previously described (37) using a reticle with a Thurlbeck grid. See supplementary text for details.

**Conscious lung function measurements by whole body plethysmography.** Rats were placed in a plethysmograph chamber (Buxco, Wilmington, NC) and left to acclimatize for 5 min. Bias flow (air removed from chamber) was set at 1 l/min. The pressure wave forms generated by respiration of the rats were analyzed by Buxco software. PenH (area under the curve; PenH AUC), peak expiratory flow (PEF), peak expiratory flow rate (PEFR), and resonant frequency (RF) were calculated.

Fig. 1. Temporal changes in gene expression in response to cigarette smoke (CS) exposure are shown. Heat maps represent hierarchical clustering of all genes present on the microarray chip from selected time points over the course of the study. Each time point is indicated above its respective heat map. The red branch lines indicate individual sham animals, and the blue branch lines represent CS-exposed rats. Blue bars represent low expression levels, red bars represent high expression levels, and yellow bars represent no change relative to normalized median gene expression values. D, day; CC, cessation cluster.
and breathing frequency were calculated on a breath by breath basis as previously described (2, 6, 17) and averaged every 30 s. All measurements were calculated over a 5-min recording period.

**Unconscious lung function measurements using forced maneuvers.** Respiratory function was measured using methods similar to those described previously (1, 4). See supplementary text for details.

**Statistical analysis.** All data are presented as means ± SE. Unless otherwise stated, statistical significance of data was determined using a two-tailed t-test with each group being treated as an independent group and significance measured only against the time-matched, air-exposed controls. A result was considered significant if \( P < 0.05 \).

**RESULTS**

**CS-induced changes in gene expression.** Principal component analysis (PCA) was used to identify the major set of expression profiles within the 340 genes identified as being significantly different between smoke- and air-exposed rats from pairwise comparisons. The tool characterizes the most abundant themes or building blocks that occur in many genes within the experiment. PCA identified nine distinct expression profiles over the course of the study (see Supplemental Fig. 1). Of the nine, two clusters were clearly either preferentially downregulated or upregulated in response to CS exposure, whereas there were four others that demonstrated gradual increases in expression.

For clustering analyses, gene chips from sham- and CS-exposed samples at each time point were clustered using the Pearson correlation as the similarity measure. This process attempts to group the most similar chips together based solely on the expression patterns and values. Figure 1 shows the results of the experimental clustering analysis. Earlier time points (1–5 days) show no separation of the two treatment groups based on global gene expression. Starting from 2 wk, the two treatment groups begin to form distinct clusters based on treatment. Once the sham and CS-exposed groups have separated at 2 wk, they remain so during the rest of the experiment. This clear pattern of differential gene expression gained in intensity over the course of the model. In the smoking cessation group, the distinction between sham- and smoke-exposed rats largely remained apart from one CS-exposed rat that grouped with the sham animals (Fig. 1).

**Pathway changes in response to smoke.** During the first week, metabolic functions, in particular genes involved in oxidative phosphorylation and glycolysis (Fig. 2 and Supplemental Fig. 2), were most significantly elevated by smoke exposure. Signal transduction gene sets were significantly reduced. Several metabolic pathways remained upregulated over the course of the study (e.g., peroxisome proliferator-activated receptor-\( \alpha \) signaling and carbohydrate metabolism);

![Fig. 2. Metabolic pathways are increased in response to smoke exposure. Heat maps represent selected genes from metabolic functions. Blue bars represent low expression levels, red bars represent high expression levels, and yellow bars represent no change relative to normalized median gene expression values. Data are shown from selected time points representing the transition from acute to chronic smoke exposure, with the last column for each group representing the smoking cessation group (Cess). W, week.](http://example.com/figure2.png)
however, by 3 wk, pathways related to immunity, defense, and proteolysis were most significantly increased (Fig. 3 and Supplemental Fig. 3). Both inflammation and stress response/detoxification genes were upregulated acutely. Genes including the rat GRO homologs, IL-1β, and nuclear factor-erythroid 2-related factor 2 (NF-E2 factor 2 or Nrf2) were increased from 3 days and stayed elevated over the course of the study. Many of these changes became more pronounced as the duration of exposures increased (Fig. 3). In particular, those genes associated with macrophage function (e.g., matrix metalloproteinase-12) and phagocytosis (e.g., CD36) progressively increased over the course of the model. This was also true for genes associated with the lymphocyte function such as Ly-6C antigen, IgG, and immunoglobulin receptor expression. By 34 wk, gene sets related to immunity and defense and B cell-mediated immunity were the most significantly increased (Fig. 3 and Supplemental Fig. 4). Major histocompatibility complex class II- and macrophage-mediated immunity pathways were also very significantly elevated. Although slightly lower than at earlier time points, inflammation and stress response/detoxification gene sets remained increased in the smoking cessation group (Fig. 3). In contrast, most the gene sets involved in metabolic processes returned to control levels (Fig. 2).

**CS-induced oxidative damage.** Immunohistochemical analysis of the rat lungs for 4HNE protein modification showed that there was no difference in the general staining pattern and intensities between the CS-exposed and sham-exposed rat lungs. However, CS-exposed lungs exhibited stronger staining in the alveolar macrophages compared with the sham-exposed lung samples (Fig. 4). In addition, in the 26-wk CS-exposed rat lung, the terminal bronchial epithelium associated with macrophage lesions displayed increased 4HNE staining. The alveolar macrophages in the 8- and 26-wk CS-exposed rat lung also appeared to be clumped together in foci around the alveolar air spaces (Fig. 4D).

**Effect of CS exposure on inflammatory mediator levels.** The neutrophil chemokines, cytokine-induced neutrophil chemoattractant-1 (CINC-1) and CINC-2 (rat GRO homologs), were increased in response to CS exposure in a biphasic fashion in both BAL fluid (Fig. 5, A and B) and lung homogenate supernatant (Fig. 5, C and D). The initial peak appeared at 3 days, followed by a small decrease in the levels of these chemokines until 4 wk. Subsequently, the second phase of CINC production occurred, peaking between 12–16 wk. The CINC-1 response returned to baseline levels after smoking cessation, whereas, in contrast, CINC-2 levels remained significantly elevated in the tissue (P < 0.05) although much lower than earlier time points.

IL-1β was also increased in response to CS, however, only in the lung homogenate supernatants (see Supplemental Fig. 5). The levels were elevated and greatest after 3 days of exposure, increasing from 173 ± 25 pg/mg tissue in sham animals to 375 ± 57 pg/mg tissue in the CS-exposed rats (P < 0.01). The levels remained significantly increased over the course of the study but returned to control levels after smoking cessation. In addition, IL-6, IFN-γ, monocyte chemoattractant protein-1, TNF-α, and IL-4 were assayed for but were below the levels of detection. Levels of IL-13 were measured and, although variable, appeared to be increased at some but not all time points (data not shown).

Effect of CS exposure on inflammatory cell infiltration. CS increased the number of neutrophils in the BAL fluid in a biphasic pattern similar to that of the neutrophil chemokines (Fig. 6A). After an initial peak at 5 days, the number of neutrophils increased again starting from 6 wk and reached their greatest levels at 34 wk, increasing from 0.5 ± 1 × 10^5 in the sham animals to 14.7 ± 2.3 × 10^5 neutrophils recovered in the BAL fluid of CS-exposed rats. A similar biphasic pattern of neutrophil infiltration was observed in the lung tissue (Fig. 6B). In both the BAL and tissue, the numbers of neutrophils decreased from their peak levels in the cessation group; however, neutrophil numbers remained elevated after smoking cessation in BAL fluid (P < 0.001).

Macrophage numbers in the BAL fluid were significantly decreased in response to CS exposure starting from 3 days, continuing over the duration of the study, and stayed significantly lower in the smoking cessation group (P < 0.05; Fig. 6C). Conversely, macrophage numbers in the tissue increased starting from 5 days and slowly but progressively increased over the course of the study (Fig. 6D). They reached a maximum...
3.7-fold increase at 34 wk ($P < 0.001$). Again, the number of macrophages in the tissue remained significantly elevated after 8 wk of smoking cessation ($P < 0.01$) although they were lower than earlier time points. No consistent increases in T lymphocytes were detected in the BAL fluid or tissue (data not shown); however, these cells appeared to be upregulated in the CS-exposed animals, primarily localizing to areas containing macrophage aggregations and vessels that were excluded from the counting method.

The most prominent inflammatory changes occurred in the alveoli, where pigmented, some binucleated, macrophages began to accumulate by 3 days. These aggregations of macrophages began to fill alveolar spaces by 3 wk, initially in those spaces adjacent to the terminal bronchioles (Fig. 7B). At 6–8 wk, the aggregates progressively spread into surrounding spaces and began to incorporate neutrophils (Fig. 6C). Many of these cells showed signs of degeneration and cell death as determined by nuclear pyknosis and debris. These inflammatory lesions progressively worsened over the duration of the study (Fig. 7D). The interalveolar walls were multilocally distended with aggregates of lymphocytes and macrophages. In addition, by 8 wk, a moderate perivascular lymphocytic inflammation was present in all CS-exposed animals (data not shown).

There were also some mild inflammatory changes around the smaller airways in the CS-exposed rats. Acutely, around the bronchioles, there was a mild intra- and subepithelial neutrophilic inflammation that decreased after 2 wk and was not

Fig. 4. A–D: lung 4-hydroxy-2-nonenal (4HNE) staining of alveolar macrophages in the rat smoking model. A representative picture of 4HNE adduct staining in alveolar macrophages is shown for sham-exposed rat lungs (A), 3-day smoke-exposed rat lungs (B), 8-wk smoke-exposed rat lungs (C), and 6-mo smoke-exposed rat lungs (D). Arrows show alveolar macrophages. E: alveolar macrophage 4HNE staining is elevated in smoke-exposed rat lungs compared with sham-exposed animals ($n = 6$). Closed circles represent smoke-exposed animals, open circles represent sham-exposed animals, and the horizontal lines indicate the mean values with $n = 6$ per group; *$P < 0.05$. 

E: Alveolar Macrophage Staining

<table>
<thead>
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Closed circles represent smoke-exposed animals, open circles represent sham-exposed animals, and the horizontal lines indicate the mean values with $n = 6$ per group; *$P < 0.05$.
present at 34 wk. There were also mild peribronchiolar lymphocytic infiltrations in some CS-exposed animals after 3 wk (data not shown).

Effect of CS exposure on the epithelium of the central airways. CS induced epithelial hypertrophy and hyperplasia (increased number of mitotic bodies) that peaked at 5 days but was subsequently attenuated thereafter (Fig. 8). These changes were also associated with a significant increase in the number of mucin-containing cells in both the trachea (Fig. 8, A–D) and the primary bronchus. The area of epithelium stained positive for mucin in both the trachea and the bronchus was increased from 3 days and progressively increased over the course of the study, reaching a plateau by 16 wk (Fig. 8E). These changes in mucus levels in the lung tissue correlated closely to the amount of mucin detected in the BAL fluid (Fig. 8F).

Effect of CS exposure on smaller airways. The acute response to CS in the epithelium was associated with an increased number of mitotic bodies found in the epithelial cells in the small airways (data not shown). The epithelium in sham animals were a single cell layer of flattened to cuboidal cells; however, in the CS-exposed rats, the epithelium was multilayered containing cuboidal to prismatic cells. The hyperplastic response began to attenuate after 3 days but remained elevated throughout the study.

After 16 wk of CS exposure, collagen deposition around the airways (other than primary bronchus) increased from...
26.9 ± 1.7 to 56.0 ± 2.5 μm (P < 0.0001; Fig. 9A). As time progressed, the amount of collagen around the airways in sham animals increased. Although there was still more collagen around the airways of CS-exposed animals, the increase was no longer significant. The amount of smooth muscle around the airways was not significantly changed compared with air-exposed controls after 16 or 34 wk (Fig. 9B).

Effect of CS exposure on alveolar structure. In the alveoli, the alveolar walls of CS-exposed rats were lined with cuboidal type II pneumocytes by 6 wk. The mean linear intercept of the alveoli of CS-exposed animals increased after 34 wk of exposures from 50.4 ± 2.0 μm in control animals to 61.7 ± 3.9 μm in CS-exposed (P < 0.05; Fig. 10, A–C). This translates into an increase of ~20%. No difference was detected between the groups that received 26 wk of either air or CS exposures (data not shown).

Effect of CS exposure on lung function. CS exposure caused very acute changes in lung function, as measured by conscious whole body plethysmography (see Supplemental Fig. 6). Small increases in PenH and decreases in PEF were detected; however, the changes were transient, and lung function returned to normal within 6 h. The increased breathing frequency in CS-exposed animals during 6-h measurements was due to differences in the exploratory behavior of the animals at this time. There were also no consistent, significant changes in forced expiratory volume in 100 ms (FEV100), forced vital capacity (FVC), or TLC as measured by unconscious forced maneuvers (see Supplemental Fig. 7).

DISCUSSION

The response to CS exposure in rat was comprised of two distinct phases: an acute phase that peaked at the end of the first week and a second, more chronic phase that started after a transcriptional shift in the gene expression pattern of the CS-exposed rats, which occurred after 2 wk of exposures. Many of the molecular changes induced by CS were progressive and present during both phases. However, most of the lung pathology associated with smoking was apparent in the chronic phase.

The major features of the acute phase included an immediate increase in the expression of metabolic gene sets and stress response genes, inflammation, and changes to the epithelium. Increased expression of metabolic gene sets was a new finding that has not been previously reported in the response to smoke. We propose that these changes are due to increased energy demands for repairing smoke-mediated damage. In addition, these pathways are critical for producing important reducing equivalents that may be needed to protect cells from the oxidizing effects of smoke. There is evidence to support the concept that energy transfer pathways and oxygen detoxification pathways coevolved and are increased in concert in response to environmental stresses such as CS (33).

Stress response genes were also increased during the acute phase. Enzymes such as heme oxygenase-1 (HO-1), thioredoxin, γ-glutamylcysteine synthetase (γ-GCS), and manganese-containing SOD (MnSOD) and the transcription factor
Nrf2 were immediately upregulated, consistent with previous reports (9). These genes appear to be critical in the response to smoke and have been implicated in the pathogenesis of COPD. HO-1 expression is upregulated in alveolar macrophages of smokers (20), and promoter polymorphisms for this gene have been identified in certain COPD patients (40). Furthermore, overexpression of HO-1 protects against lung injury in elastase and CS models (31, 36). HO-1 and other antioxidant enzymes are regulated by Nrf2 (5), which is essential for protection against CS-induced inflammation and emphysema (29).

The inflammation in this acute phase was primarily neutrophilic in nature. The observed decrease in BAL macrophage numbers is likely due to the activation of these cells. In addition, oxidant modification to matrix proteins by CS extract has also been proposed to enhance the adhesion of macrophages to matrix (16). Another basic feature of the acute response to CS was the epithelial thickening and goblet cell metaplasia, which peaked after 5 days of exposures. Subsequent to this acute phase, components of the innate, host defense response significantly decreased but did not completely resolve. Conversely, tissue macrophage numbers began to increase. The mechanisms underlying this transition to the...
second, more chronic phase in the response to smoke is unknown; however, we propose two hypotheses to explain these observations that may occur independently or collectively. First, alveolar macrophages, which migrate into the lung to resolve inflammation, are damaged by oxidants and may become overloaded with particulate matter with continual exposure to CS. These changes can result in reduced migration and enhanced activation of macrophages (25, 26). This may trigger the chronic inflammatory response that is observed following 2 wk of exposures. This hypothesis is consistent with the observations made in this model, with increased 4HNE staining in alveolar macrophages that are particle-laden and accumulating in large aggregates in the alveoli and alveolar ducts by 2 wk.

Second, ultrafine particles may localize to the macrophage mitochondria inducing structural damage (18) thereby reducing the metabolic capacity needed for protection against CS exposure (33). This damage to the metabolic machinery leaves the cell no longer capable of adapting to meet the added demands of further CS exposures. This may account, at least in part, for the “transcriptional shift” that occurs in gene expression patterns after 2 wk of CS exposures, and we propose that these changes are responsible for initiating the chronic response to smoke. In summary, we hypothesize that eventually the system becomes overwhelmed with repeated oxidant and particulate challenges, resulting in metabolic insufficiency and subsequently a greater oxidant burden leading to greater inflammation.

The second chronic wave of inflammation, oxidant damage, and mucus production was progressive and greater than the initial acute response. As shown previously, the inflammation shifted from a primarily neutrophilic response to one that had greater macrophage involvement (12, 27). Lymphocytes were present in these aggregates, and markers of the adaptive immune response (e.g., B cell-mediated immunity pathway, Ly-6C antigen and IgG chains) were increased in the chronic phase; however, the presence of lymphocytes were not as apparent as in smoking models reported in other species such as the mouse (7).

Associated with this second wave of inflammation were the start of structural changes in the lung consistent with those observed in smokers and COPD patients including collagen deposition around the airways and macrophage aggregation. There has previously only been one other report that we know of demonstrating a change in airway collagen in rats in response to CS exposure (30). The increase in airway collagen was no longer significant at 34 wk due to the increased level of collagen in the air-exposed controls. After 34 wk of exposure, there was a significant increase in the mean linear intercept of the alveolar spaces in CS-exposed rats. The failure to see greater structural damage to the lung and subsequent changes to lung function is possibly related to a lack of any viral and/or bacterial infection in this model system.

In the smoking cessation group, the changes induced by CS exposure were resolving; however, many of the inflammatory and antioxidant genes remained significantly elevated and were associated with increased numbers of inflammatory cells as has been documented in COPD patients (13). Despite this, other phenotypic changes (e.g., mucus production) had returned to control levels.

One limitation to this study is that the microarray experiments were conducted using RNA generated from whole lung rather than single cell types. Therefore, changes in expression profiles may reflect changes in cellular constituency of the lung at each time point. Ideally, it would be useful to analyze the expression changes occurring in a more homogenous cell population using laser capture microdissection. However, this study is strengthened by the fact that samples from individual rats were used for each time point in contrast to previously published work (9) where pooled samples were used. The use of individual samples allowed us to differentiate CS- and air-exposed groups in a more significant and comprehensive fashion. In addition, for the first time in a rodent smoking model, we used both pathway- and gene-centric analysis of the microarray data in combination with pathological observations. This approach placed these changes in a broader biological context and led to the identification of changes in biochemical processes previously not associated with the response to CS inhalation or COPD.

In summary, this study has provided new insights into the molecular pathways activated in response to CS and possibly involved in the pathogenesis of COPD. After a transient acute response where metabolic pathways appeared most significantly changed, a transcriptional shift in the gene expression pattern after 2 wk appears to initiate the chronic phase of the response to smoke inhalation. This shift may be due to changes in metabolic capacity and/or macrophage function, similar to the situation found in human (10, 12). Chronic CS inhalation leads to greater activation of immunity and defense pathways and recapitulated many of the phenotypic changes observed in COPD patients. These include oxidative damage to macrophages, a chronic, low-grade inflammation consisting of neutrophils, macrophages, and lymphocytes, epithelial damage, mucus hypersecretion, airway fibrosis, and emphysema.

These data suggest that the rat model may provide insights into the molecular mechanisms underlying the response to chronic smoking that lead to the destructive lung pathologies observed in COPD patients. In addition, using “Omics” technologies to characterize both animal models and clinical samples may help identify the gaps and parallels in these systems, as well as uncover prospective biomarkers, both of which are vital for identifying and measuring the efficacy of drug candidates for COPD.

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