Particulate Matter in Cigarette Smoke Alters Iron Homeostasis to Produce a Biological Effect

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***Rationale*: Lung injury after cigarette smoking is related to particle retention. Iron accumulates with the deposition of these particles.**

***Objectives*: We tested the postulate that (*1*) injury after smoking correlates with exposure to the particulate fraction of cigarette smoke, (*2*) these particles alter iron homeostasis, triggering metal accumulation, and (*3*) this alteration in iron homeostasis affects oxidative stress and inflammation.**

***Methods*: Rats and human respiratory epithelial cells were exposed to cigarette smoke, filtered cigarette smoke, and cigarette smoke condensate (the particulate fraction of smoke), and indices of iron homeostasis, oxidative stress, and inflammatory injury were determined. Comparable measures were also evaluated in nonsmokers and smokers.**

***Measurements and Main Results*: After exposure of rats to cigarette smoke, increased lavage concentrations of iron and ferritin, serum ferritin levels, and nonheme iron concentrations in the lung and liver tissue all increased. Lavage ascorbate concentrations were decreased, supporting an oxidative stress. After filtering of the cigarette smoke to remove particles, most of these changes were reversed. Exposure of cultured respiratory epithelial cells to cigarette smoke condensate caused a similar accumulation of iron, metal-dependent oxidative stress, and increased IL-8 release. Lavage samples in healthy smokers and smoking patients with chronic obstructive pulmonary disease revealed elevated concentrations of both iron and ferritin relative to healthy nonsmokers. Lavage ascorbate decreased with cigarette smoking. Serum iron and ferritin levels among smokers were increased, supporting systemic accumulation of this metal after cigarette smoke exposure.**

***Conclusions*: We conclude that cigarette smoke particles alter iron homeostasis, both in the lung and systemically.**

Keywords: smoking; ferritin; oxidants; chronic obstructive pulmonary disease

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The mechanism(s) for tissue injury after cigarette smoking is not known. Reasons for the persistence of risk for disease after cessation of smoking similarly is not recoganized.

What This Study Adds to the Field

This investigation supports a mechanism of tissue injury after disruption of iron homeostasis (both in the lung and systemically) by cigarette smoke particles. Accumulated iron then catalyzes oxidative stress and biological effect.

Cigarette smoking is one of the 10 greatest contributors to global death and disease (1). The increased risk of lung injury due to smoking (e.g., chronic obstructive pulmonary disease [COPD] and cancer) frequently does not diminish after smoking cessation and persists in exsmokers (2, 3). The basic cellular and molecular events underlying the biological effects of cigarette smoke and reasons for their persistence despite cessation of the exposure are not fully appreciated.

In a burning cigarette, temperatures in the combustion zone (800–950°C) result in a complete pyrolysis of tobacco (4). Immediately downstream, a rapid drop in temperature (200–600°C) and a lack of oxygen allow for an incomplete combustion. Subsequently, a complex aerosol is generated, which includes condensed liquid droplets (the particulate fraction or tar) suspended in a mixture of volatile and semivolatile compounds and combustion gases (the gas fraction). Smoking one cigarette exposes the human respiratory tract to between 10,000 and 40,000 μg particulate matter (PM) (5). These particles have a mean diameter (<1 μm) that allows a high rate of deposition in the human lung (6). In humans, lung injury after cigarette smoking appears to be particle-related, because tissue destruction is immediately adjacent to the retained particle (Figure 1A).

The composition of cigarette smoke PM is comparable to that of other particles generated through combustion of carbonaceous material, with incomplete oxidation producing oxygen-containing functional groups (e.g., carboxylates, esters, and phenolic hydroxides) in greatest concentration at the surface (6–8). These oxygen-containing functional groups undergo proton dissociation at physiologic pH, which introduces a negatively charged solid–liquid interface into the lung. As a result of its electropositivity, Fe^{3+} has a high affinity for such oxygen-donor ligands (9), and this metal is subsequently complexed by cigarette smoke particles (10). In support of this coordination complex occurring *in vivo*, particles retained in the lower respiratory tract of cigarette smokers accumulate iron (Figure 1B) (8). After complexation of the metal by functional groups, a lack of pliancy by the inflexible particle surface predicts that placement of electrons into the symmetrically located coordination sites of iron would be incomplete; this allows participation of the complexed metal in electron transport and catalysis of oxidants. Therefore, metal complexation by functional groups at the surface of the retained cigarette smoke PM will catalyze production of damaging oxidants in the environment immediately adjacent to the particle.

The source of the iron that accumulates in the lung after exposure to cigarette smoke has not been identified. The metal could originate from either the cigarette or the host. Tobacco has been reported to contain 440–1,150 μg iron/g (11). Only a small amount of this iron (0.1%) enters mainstream smoke (11), and this quantity is not considered significant. Alternatively, specific host sources of iron (e.g., a labile iron pool) (12) could be bound

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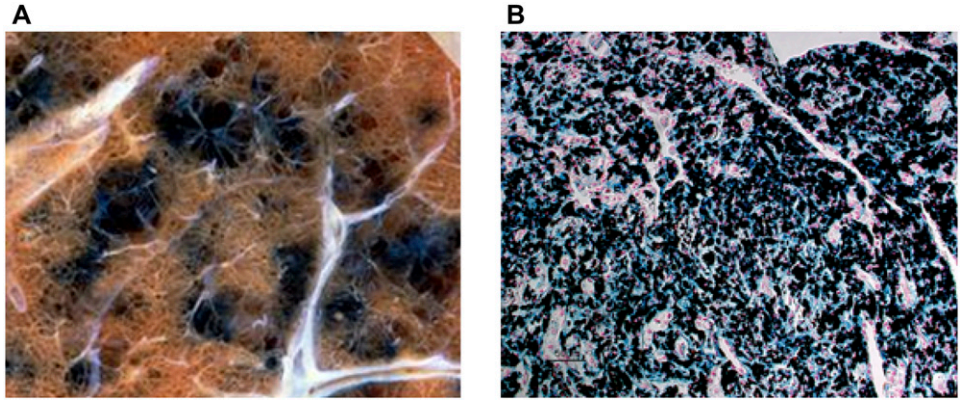

Figure 1. Lung injury after cigarette smoking shows a relationship with both particles and iron. Lung collected at autopsy showed a correlation between the retention of cigarette smoke particles and destruction of lung parenchyma (i.e., bullous formation in this emphysematous patient), (*A*) photomicrograph at a magnification ~10× (courtesy of Dr. Phil Pratt, formerly of Duke University Medical Center, Durham, NC). (*B*) A photomicrograph demonstrated that, comparable to tissue destruction, iron in the lung of a smoker is also particle-associated (Perls' Prussian blue stain with the iron staining *blue*; magnification, ~100×).

by the PM surface after its deposition in the lung. Such complexation of host iron by cigarette smoke particles is likely to alter iron homeostasis, both in the lung and systemically. Metal accumulation would result in oxidative stress, which would precipitate an inflammatory response. Delineating the alteration in host iron homeostasis, the subsequent accumulation in metal and oxidant generation, and the consequent inflammation after exposure to cigarette smoke PM would contribute to understanding both the health effects of smoking and their persistence after cessation; these will be observed in exsmokers if the particle is responsible for the biological effect and injury, because the particles are retained for prolonged periods of time and, perhaps, for the lifetime of the individual.

We tested the postulate that: (*1*) injury after smoking correlates with exposure to the particulate fraction of particular matter; (*2*) cigarette smoke particles alter iron homeostasis, triggering an accumulation of the metal; and (*3*) this alteration in iron homeostasis affects oxidative stress and inflammation.

METHODS

Animal Exposures

The University of California at Davis Institutional Animal Care and Use Committees reviewed and approved all procedures on animals. Male Wistar rats were exposed to air, cigarette smoke, and filtered (to remove particles) cigarette smoke (n = 10/exposure) for 6 hours/day on 3 consecutive days. Cigarette smoke and filtered cigarette smoke exposures initially shared a single pathway, which was then divided for delivery into two chambers containing rats. After the split, the pathway for filtered cigarette smoke exposure included a Microguard 99 Air Filter (Airguard Industrial, Carona, CA). Levels of total suspended particulates during the exposures to cigarette smoke and filtered smoke (mean ± SD) were 80.94 ± 5.70 mg/m^3 and 0.20 ± 0.02 mg/m^3, respectively. Carbon monoxide concentrations during the exposures to cigarette smoke and filtered smoke (mean ± SD) were 232 ± 9 ppm and 201 ± 13 ppm, respectively.

Specimens acquired on Day 4 included tracheal lavage with phosphate buffered saline (PBS) (n = 6/exposure) acquired as previously described (13), blood (n = 6/exposure), unfixed lung and liver (n = 4/exposure), and inflation-fixed lung (10% formalin; n = 4/exposure).

Indices of Inflammation and Injury

Cigarette smoke exposure causes an inflammatory lung injury in animals (14). A modified Wright's stain (Diff-Quick stain; American Scientific Products, McGaw Park, IL) was used to quantify lavage neutrophils, and values were expressed as the percentage of total cells recovered. Lavage protein and albumin concentrations were employed as indices of lower respiratory injury; these were determined using the Pierce Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) and an immunoprecipitin assay (Diasorin, Stillwater, MN), respectively.

Indices of Iron Homeostasis

Lavage and serum iron concentrations were determined using a colorimetric, enzymic method (Sigma Diagnostics, St. Louis, MO). Ferritin concentrations were measured using an enzyme immunoassay (Microgenics Corporation, Concord, CA). Transferrin concentrations were analyzed using an immunoprecipitin analysis (INCSTAR Corp., Stillwater, MN).

Nonheme iron concentrations in cells and resected lung and liver tissues were quantified using inductively coupled plasma optical emission spectroscopy (Model Optima 4300D, Perkin Elmer, Norwalk, CT) operated at a wavelength of 238.204 nm (15).

Lungs were inflation fixed with 10% formalin for 24 hours and then transferred to 70% ethanol. Immunohistochemical staining for ferritin and divalent metal transporter (DMT) 1 was accomplished as previously described (16).

Measurement of Antioxidants

Levels of ascorbate, urate, and total glutathione in acellular lavage fluid were measured to describe the oxidative stress in the lower respiratory tract after animal and human exposure to cigarette smoke (17, 18).

Macrophage Inflammatory Protein-2 and IL-8 Concentrations

Concentrations of macrophage inflammatory protein-2 (MIP-2) and IL-8 in acellular lavage fluid and cell media were measured using ELISA kits (R&D Systems, Minneapolis, MN).

Respiratory Epithelial Cell Exposures

To better define cellular changes in iron homeostasis after exposure to cigarette smoke particles, respiratory epithelial cells were incubated with either PBS or 25 μg/ml cigarette smoke condensate (CSC) (Murty Pharmaceuticals, Lexington, KY) in PBS. CSC is the particulate fraction of cigarette smoke. There were 3.1 ± 0.2 ppm Fe in the CSC (measured by inductively coupled plasma optical emission spectroscopy after nitric acid digestion of the CSC at 70°C for 24 h). BEAS-2B cells, an immortalized line of normal human bronchial epithelium, were grown to 90% confluence on uncoated, plastic, 12-well plates in keratinocyte growth medium (Clonetics, Walkersville, MD). Cytotoxicity of CSC was measured using lactate dehydrogenase release and methylthiazoletetrazolium reduction.

Ferritin and IL-8 Concentrations

BEAS-2B cells were exposed to either PBS or 25 μg/ml CSC in PBS for 24 hours. IL-8 concentrations in cell media were measured using ELISA. Cells were washed, scraped into 1.0 ml PBS, and disrupted using four passes through a 25-gauge needle. The concentrations of ferritin were quantified in this lysate.

Reverse Transcription–Polymerase Chain Reaction

BEAS-2B cells were dislodged from wells with scrapers (Costar) into guanidine isothiocyanate and sheared with four passes through a 25-gauge

syringe. Quantitative polymerase chain reaction (PCR) was performed using Taqman polymerase with detection of SYBR Green fluorescence on an ABI Prism 7,700 Sequence detector (PE Biosystems, Foster City, CA). DMT1 mRNA levels were normalized using the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. The following sequences were employed: DMT1: sense GGAGCAGTG GCTGGATTTAAGT; antisense CCACTCCCAGTCTAGCTGCAA; probe TGGATCCTTCTGTTGGCCACCCTTGT; GAPDH: sense GA AGGTGAAGGTCGGAGTC; antisense GAAGATGGTGATGGGA TTTC; probe CAAGCTTCCCGTTCTCAGCC.

Cell Oxidant Generation Measured by Dichlorodihydrofluorescein Fluorescence

Oxidant generation by BEAS-2B cells was determined using dichlorodihydrofluorescein (DCF) fluorescence. The cells were loaded for 30 minutes with 10 μM dichlorodihydrofluorescein diacetate in keratinocyte growth medium and exposed to either PBS or 25 μg/ml CSC in PBS. Fluorescence was measured on a spectrofluorimeter with excitation and emission set at 485 nm and 535 nm respectively. Oxidant generation was expressed as the ratio of fluorescence relative to cells with no exposure to CSC immediately after loading with DCF.

Bronchoalveolar Lavage of Healthy Volunteers and Patients

Healthy, nonsmoking and healthy smoking volunteers (both 18–40 yr of age) underwent fiberoptic bronchoscopy with bronchoalveolar lavage (BAL). The protocol and consent form were approved by the University of North Carolina School of Medicine Committee on the Protection of the Rights of Human Subjects. The fiberoptic bronchoscope was wedged into a segmental bronchus of the lingula and then the right middle lobe. Aliquots of sterile saline were instilled and immediately aspirated, centrifuged, and stored at −70°C.

BAL was obtained from patients with chronic COPD included in the Feasibility of Retinoids for the Treatment of Emphysema trial (19). The institutional review boards of participating centers sanctioned the trial. All patients were active smokers, met the criteria for Global Obstructive Lung Disease stage-IIB COPD (20), and had evidence of emphysema on computed tomography of the chest. After consent was obtained, fiberoptic bronchoscopy was performed. BAL was performed by instilling saline solution into either the medial or lateral segment of the right middle lobe, followed by aspiration. The fluid was centrifuged and stored at −70°C.

Before assays of human lavage samples for iron homeostasis, oxidative stress, and IL-8, comparability was confirmed by quantifying urea nitrogen concentrations (Thermo Electron, Louisville, CO).

Indices of Systemic Iron Homeostasis

In order to evaluate for systemic alterations in iron homeostasis among nonsmokers and smokers, data from the National Health and Examination Survey III (conducted during 1988–1994) was analyzed. This included persons aged 20 years and older in whom data on age, race, sex, serum iron, serum ferritin, and transferrin saturation were available. Of these 13,941 persons, 2,944 were excluded because they reported a respiratory infection at either the time of or within the 3 weeks before the interview. Individuals were categorized as either "nonsmoker" or "smoker." A nonsmoker was defined as a person who reported smoking less than 100 cigarettes during their lifetime, and whose serum cotinine concentration was less than 1.0 ng/ml. A smoker was defined as a person who reported smoking cigarettes for at least a 1-year duration at the time of interview. Records of 3,746 persons who did not fit these definitions of nonsmoker and smoker were eliminated from the database, for a final sample size of 7,251 adults. Geometric means of serum iron, ferritin, and transferrin saturation are reported by age category. Linear regression models for these three measures were also obtained using sex, age, race, and smoking status as independent variables. All analyses were performed using SAS statistical software (SAS Institute Inc., Cary, NC).

Statistical Analysis

Unless otherwise specified, data are expressed as mean value ± SE. Differences between multiple groups were compared using analysis of variance. The *post hoc* test employed was Scheffé's test. Two-tailed tests of significance were employed. Significance was assumed at a P value less than < 0.05.

RESULTS

Relative to rats exposed to air, those exposed to cigarette smoke had a greater percentage lavage neutrophils, supporting an incursion of inflammatory cells into the lower respiratory tract (Table 1). Concentrations of MIP-2, a cytokine pertinent to neutrophil influx into the rat lung, were similarly increased after cigarette smoke exposure (Table 1). This inflammatory influx was accompanied by lung injury reflected by significant elevations in lavage protein and albumin concentrations (Table 1). Removal of particles by filtering eliminated almost all changes in lavage neutrophils, MIP-2, protein, and albumin after exposure to cigarette smoke.

Exposure of rats to cigarette smoke led to elevated lavage iron and ferritin concentrations (Figures 2A and 2B, respectively). Animals exposed to filtered smoke had modest elevations in iron and ferritin, but these were significantly lower relative to those after cigarette smoke. Similar to iron and ferritin, lavage transferrin concentrations were elevated after cigarette smoke exposure, but not after filtered smoke exposures (Figure 2C). Nonheme iron concentrations in resected lung tissue were significantly elevated after exposure to cigarette smoke (Figure 2D). As with iron and ferritin, exposure to filtered smoke modestly increased nonheme iron concentrations above control values, but these levels were significantly lower than in animals exposed to cigarette smoke (Figure 2D).

In support of an oxidative stress caused by particles in cigarette smoke, lavage ascorbate concentrations decreased after exposure to cigarette smoke, but not after filtered smoke (animals exposed to: air, 0.5 ± 0.1 μg/ml; cigarette smoke, 0.2 ± 0.0 μg/ml; filtered smoke, 0.4 ± 0.1 μg/ml). Lavage urate and glutathione concentrations did not change with exposure to either cigarette smoke or filtered smoke (data not shown).

The presence of an iron-responsive element allows for a rapid post-transcriptional increase in ferritin expression by iron (21). Immunohistochemistry for ferritin confirmed an accumulation of this storage protein relative to air exposure in the rat lung after cigarette smoke, but there was little change in its expression after filtered smoke (Figures 3A–3C). After cigarette smoke exposure, airway epithelial cells, endothelial cells, alveolar epithelium, and macrophages all showed increased staining for ferritin. In the lung, DMT1 similarly increases with iron availability, and this protein can coordinate with ferritin in the uptake and storage of this metal (22). Similar to ferritin, expression of the metal transporter, DMT1, increased after cigarette smoke exposure, but showed little change after filtered smoke (Figures 3D–3F).

In animals, systemic iron homeostasis can be assessed by measuring levels of serum iron, ferritin, and transferrin and liver

TABLE 1. NEUTROPHILS AND CONCENTRATIONS OF MACROPHAGE INFLAMMATORY PROTEIN-2, TOTAL PROTEIN, AND ALBUMIN IN RAT LAVAGE FLUID

	Exposure		
	Air	Cigarette Smoke	Filtered Smoke
Neutrophils, %	0 ± 0	30 ± 9*	8 ± 6†
MIP-2, pg/ml	5 ± 2	22 ± 7*	6 ± 4
Protein, μg/ml	149 ± 15	228 ± 15*	157 ± 19
Albumin, μg/ml	34 ± 5	49 ± 7*	36 ± 8

Definition of abbreviation: MIP = macrophage inflammatory protein.

* Significantly different compared with animals exposed to air.

† Significantly different compared with animals exposed to both air and cigarette smoke.

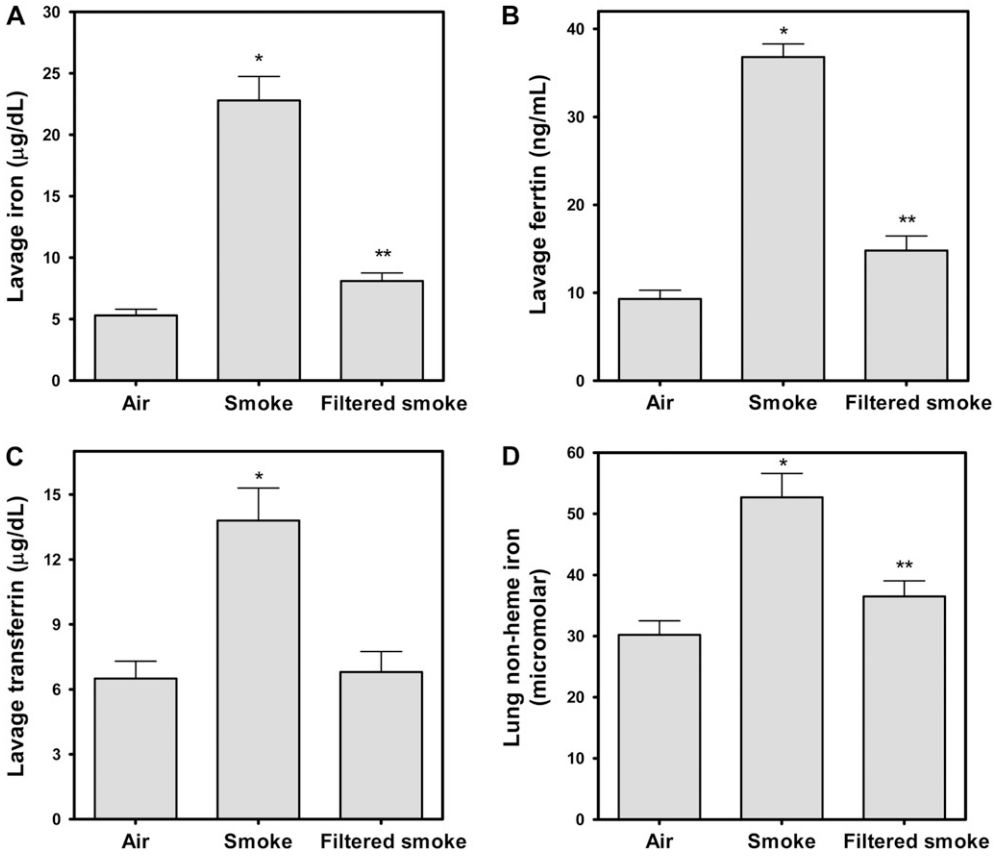

Figure 2. Iron homeostasis in the lung was altered by exposure to cigarette smoke. Animals were exposed to air, cigarette smoke, or filtered cigarette smoke to remove particles (n = 6/exposure) for 6 hours/day on 3 consecutive days. Tracheal lavage, obtained on Day 4, revealed increased concentrations of iron (*A*), ferritin (*B*), and transferrin (*C*) after exposure to cigarette smoke. These elevations appeared to be dependent on particle exposure, as filtering significantly decreased all values. Comparable to lavage endpoints of iron homeostasis, lung nonheme iron concentrations were elevated after smoke exposure, and this decreased with filtering the smoke (*G*). *Increased relative to air exposure; $P < 0.05$. ** Significant differences relative to both air and cigarette smoke exposure; $P < 0.05$. Data presented are means ± SEM.

nonheme iron concentrations. Serum iron and transferrin levels decreased in rats exposed to cigarette smoke (Figures 4A and 4C), whereas ferritin increased (Figure 4B). Relative to air, no differences were observed in these indices after exposure to filtered smoke (Figure 4A–4C). Similarly, liver nonheme iron concentrations increased after cigarette smoke, but did not change with filtered smoke (Figure 4D).

In *in vitro* investigation, BEAS-2B cells exposed to 25 μg/ml CSC for 24 hours showed no evidence of cytotoxicity; there was no significant change in supernatant lactate dehydrogenase concentrations or cell methylthiazoletetrazolium reduction. Compared with media alone, 24 hours of CSC exposure caused nonheme iron to accumulate in the BEAS-2B cells (Figure 5A). Cell ferritin concentrations similarly increased after 24 hours of incubation with CSC (Figure 5B). Further elevations in both nonheme iron concentrations and ferritin in the BEAS-2B cells were observed with 24-hour cell incubation, which included 100 μM ferric ammonium citrate (Figures 5A and 5B); these increases in cell nonheme iron and ferritin concentrations were greater in those cells exposed to CSC. Transferrin values could not be quantified (these were below the limits of detection by immunoassay). Through the use of reverse transcriptase–PCR, RNA for the transmembrane metal transporter, DMT1, was increased several fold after 4-hour 25 μg/ml CSC exposure (DMT1/GAPDH values of 0.9 ± 0.3 and 3.4 ± 1.2 for PBS and CSC exposures, respectively). This supports a role for DMT1 in metal transport and accumulation after CSC exposure. DCF fluorescence demonstrated a generation of reactive oxygen species by the respiratory epithelial cells before any exposure. Incubation of BEAS-2B cells with 25 μg/ml CSC caused an increase in DCF fluorescence signal within minutes (Figure 5C). Inclusion of 50 μM deferoxamine, a metal chelator, in the incubation diminished DCF fluorescence in cells exposed to CSC, supporting a role for iron in oxidant generation after exposure to cigarette smoke particles. IL-8 release

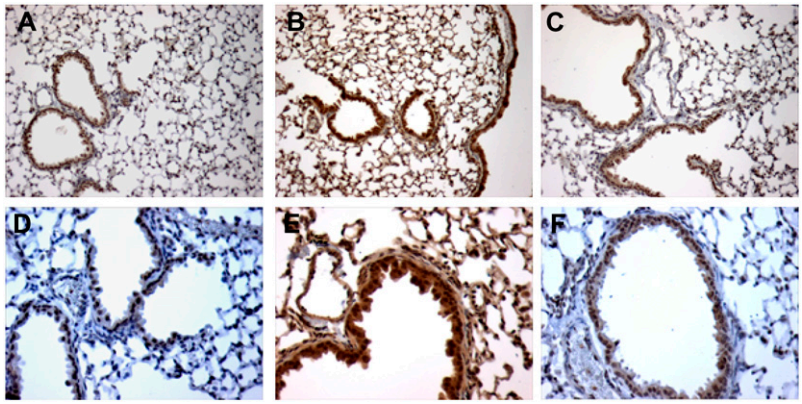

Figure 3. Ferritin and divalent metal transporter (DMT) 1 expression increased with exposure to cigarette smoke. Rats were exposed to air, cigarette smoke, or filtered cigarette smoke to remove particles (n = 4/exposure) for 6 hours/day on 3 consecutive days. On Day 4, the lungs were inflation fixed and sectioned. Immunohistochemistry for ferritin (*A–C*) demonstrated that, relative to rats exposed to air (*A*), animals exposed to cigarette smoke (*B*) increased expression of ferritin (magnification, ~100×; the ferritin stains *brown* to *red*). Filtering of the smoke with removal of particulate matter diminished the expression of this storage protein (*C*). Immunohistochemistry for DMT1 (*D–F*) showed similar results, with cigarette smoking (*E*) elevating expression relative to air exposure (*D*), whereas filtered smoke (*F*) decreased such expression.

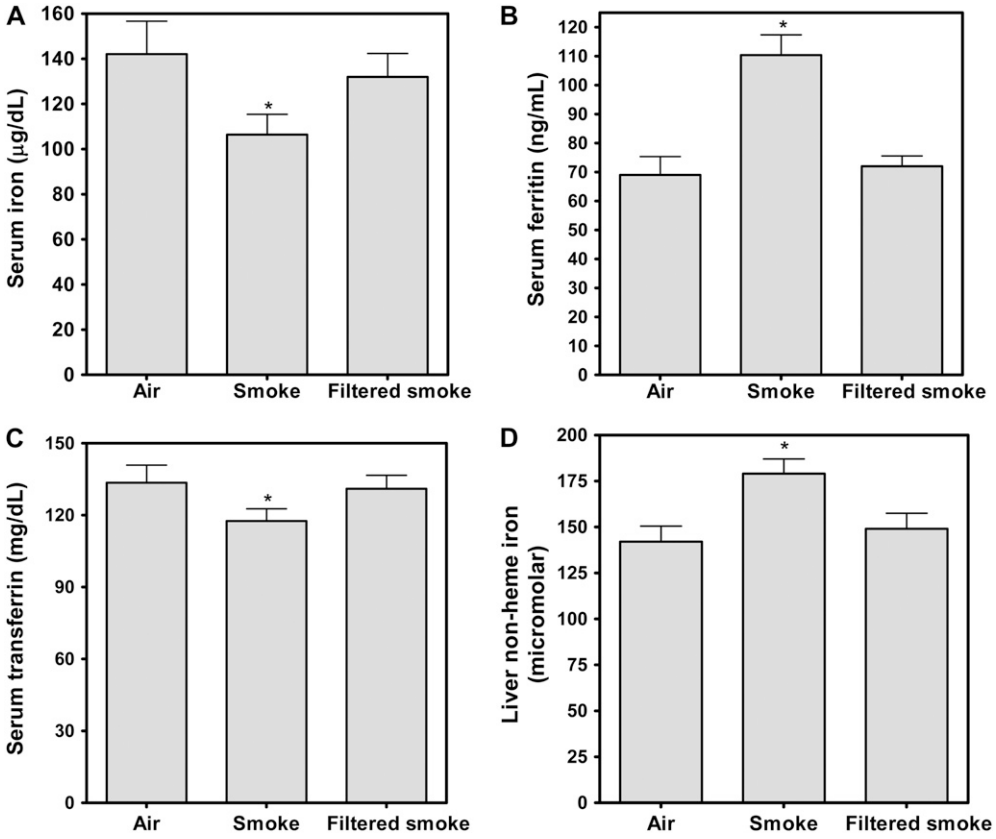

Figure 4. Systemic iron homeostasis was altered after exposure to cigarette smoke. Wistar rats were exposed to air, cigarette smoke, or filtered cigarette smoke to remove particles (n = 6/exposure) for 6 hours/day on 3 consecutive days and blood taken on Day 4. Serum iron (*A*), ferritin (*B*), and transferrin (*C*) concentrations confirmed altered iron homeostasis after cigarette smoke exposure. Removal of particles from the smoke reversed these changes. Liver nonheme iron concentrations were elevated after smoke exposure (*D*). Filtering the smoke returned liver nonheme iron concentrations to values equivalent to those after air exposure. *Significant differences relative to air exposure; $P < 0.05$. Data presented are means ± SEM.

by BEAS-2B cells after exposure to CSC was measured as a marker of inflammation. IL-8 concentrations in the cell supernatant were significantly increased after 24 hours of incubation with 25 μg/ml CSC (Figure 5D). Deferoxamine decreased IL-8 release, supporting an involvement of iron in inflammatory events after CSC exposure.

Lavage samples were obtained by bronchoscopy in 50 healthy nonsmokers, 20 healthy smokers, and 44 smokers diagnosed with COPD. Relative to healthy nonsmokers, healthy smokers included a greater number of males, whereas patients with COPD were older (Table 2). Lavage urea nitrogen values showed no significant differences (1.1 ± 0.2, 1.3 ± 0.3, and 1.3 ± 0.3 mg/dl for healthy nonsmokers, healthy smokers, and patients with COPD, respectively). Similarly, lavage total protein and albumin concentrations were not significantly different between the study groups (total protein was 85 ± 15 μg/ml, 94 ± 19, and 79 ± 24 in healthy nonsmokers, healthy smokers, and patients with COPD, respectively, whereas the albumin was 19 ± 5, 23 ± 4, and 17 ± 3 μg/ml, respectively). Lavage iron and ferritin concentrations were significantly increased in healthy smokers and patients with COPD (Figures 6A and 6B); levels of both were greatest among patients with COPD. Lavage transferrin levels were significantly decreased in smokers, and further decreased in smokers with COPD (Figure 6C). Iron staining of lavage cell pellets showed sideromacrophages in the lungs of healthy smokers (Figure 7B), but not in those from healthy control subjects (Figure 7A), confirming metal accumulation intracellularly. Similarly, staining of the lavage cell pellets for ferritin revealed greater expression of this metal storage protein in cells from smokers (Figures 7C and 7D). In order to evaluate for oxidative stress in smokers' lungs, lavage concentrations of ascorbate, urate, and glutathione were measured. Lavage ascorbate concentrations were significantly decreased in healthy smokers (0.3 ± 0.2 and 0.1 ± 0.1 μg/ml in healthy nonsmokers and healthy smokers, respectively), whereas lavage urate and glutathione concentrations demonstrated no change. Finally, lavage IL-8 concentrations were 48 ± 17, 97 ± 42, and 111 ± 45 pg/ml in healthy nonsmokers, healthy smokers, and patients with COPD, respectively, supporting an inflammatory environment in the lungs of healthy smokers and patients with COPD.

In order to delineate changes in systemic iron homeostasis with cigarette smoking, National Health and Examination Survey III data were evaluated. The number of nonsmokers and smokers was 4,473 and 2,778, respectively. Mean values (±SD) of serum iron and ferritin levels and transferrin saturation are reported, and these demonstrated significant increases among smokers relative to nonsmokers (Figures 8A–8C). Significance of cigarette smoking in each of these three serum measures of iron homeostasis was confirmed in regression models (with $F = 29.4$, $P < 0.0001$; $F = 20.6$, $P < 0.0001$; and $F = 22.9$, $P < 0.0001$, respectively) using sex, age, and race as independent variables. These results support a systemic accumulation of iron with cigarette smoking. Differences between nonsmokers and smokers in all three indices were greatest among those less than 50 years of age.

DISCUSSION

Prior studies have demonstrated increased lavage iron concentrations in smokers (23, 24). Accumulation of this metal in airway and alveolar macrophages, proportional to the frequency and duration of cigarette smoking, has also been described among smokers (25–27). In our current study, animal exposures to cigarette smoke increased lavage iron concentrations, supporting an accumulation of the metal in the lower respiratory tract. Filtering the cigarette smoke showed that these changes were associated with particle exposure. Cigarette smokers also had increased lavage iron concentrations. In addition to extracellular

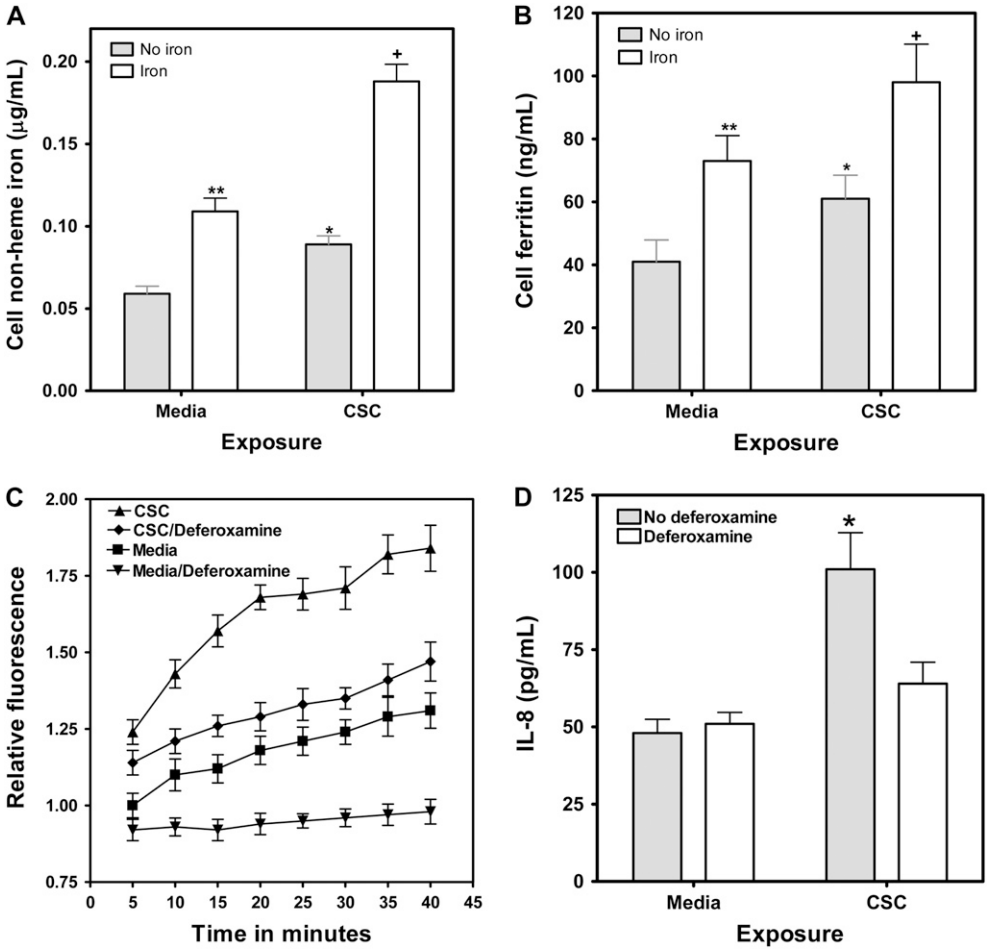

Figure 5. Cell iron, oxidative stress, and inflammatory mediator release increased with exposure to cigarette smoke condensate (CSC). Cell iron (*A*) and ferritin (*B*) concentrations were increased after 24 hours incubation with 25 μg/ml CSC. Inclusion of 100 μM ferric ammonium citrate in the media further increased both cell iron and ferritin concentrations after 24-hour incubation; elevations of both were significantly greater in coexposures of CSC and ferric ammonium citrate. Oxidant generation was also increased after CSC exposure (*C*). This increase in oxidant generation by CSC was inhibited by including 50 μM deferoxamine, a metal chelator, in the incubation. Finally, IL-8 release by the BEAS-2B cells was elevated after 24-hour exposure to 25 μg/ml CSC (*D*). Inclusion of 50 μM deferoxamine decreased the release of this inflammatory mediator. *Increased relative to media exposure; $P < 0.05$. **Increased in cells grown without additional iron. Data presented are means ± SEM.

concentrations, lavage cell pellets from smokers demonstrated increased staining for iron. Human respiratory epithelial cells exposed to CSC had elevated nonheme iron concentrations. Lung nonheme iron concentrations increased in those animals exposed to cigarette smoke, but not to filtered smoke. Collectively, these results support alterations in iron homeostasis leading to metal accumulation after cigarette smoke exposure that are dependent on PM in the smoke.

Cells, tissues, and the living system can respond to elevated concentrations of available iron by attempting to sequester the metal using ferritin (28). DMT1 may transport the metal intracellularly to facilitate storage (22). Cigarette smoke exposure of the rat increased both ferritin concentrations in lavage and its expression in lung cells; these responses were particle-dependent. Increased DMT1 expression in the lung also followed exposure of the rat to cigarette smoke particles. Similarly, cell exposures to CSC increased ferritin concentrations and DMT1 RNA. Finally, ferritin concentrations in lavage and cell pellets were elevated in smokers relative to nonsmokers. These increases in ferritin and DMT1 in lung samples support a coordinated response of the two proteins in the lower respiratory tract to iron accumulated in excess of metabolic requirements after exposure to cigarette smoke PM.

Even with transport by DMT1 and storage in ferritin, elevations of cell iron must be limited to avoid oxidative damage. Cell and tissue release of the metal is required, and this is likely to involve systemic transport (27). Serum ferritin and liver nonheme iron concentrations increased in animals exposed to cigarette smoke; these changes were particle dependent. In human smokers, serum iron, ferritin, and transferrin saturation were all elevated relative to nonsmokers. These results are comparable to those of a prior investigation (29), and may reflect a transport of the metal from the lung, not only to more secure sites of storage, such as the reticuloendothelial system, but to many organs of the body (30). Serum ferritin reflects total stored iron concentration, and its increase with cigarette smoking suggests an accumulation of the metal in smokers and overabundance relative to metabolic needs.

Transferrin is used to meet the metabolic needs of the cell for iron. It was anticipated that concentrations of this transport protein (in both the lavage and blood) would diminish with elevated iron levels after cigarette smoking. Measurement of this transport protein in the lavage of nonsmokers, smokers, and patients with COPD revealed decrements corresponding to the elevated metal availability in the lung after cigarette smoke exposure. However, serum transferrin (along with serum iron) in the animal model decreased after such exposure. The acute-phase response after the animal exposure to cigarette smoke would decrease both serum transferrin and iron, and this would confound the study of changes in metal homeostasis directly

TABLE 2. AGE, SEX, AND SMOKING CHARACTERISTICS OF THOSE HAVING BRONCHOSCOPY WITH LAVAGE

Status	Age (*Yr*)	Female (%)	Smoking (*Pack-Years*)
Healthy nonsmokers	24 ± 3	44	0
Healthy smokers	27 ± 6	10	8 ± 3
Smokers with COPD	66 ± 7	42	58 ± 29

Definition of abbreviation: COPD = chronic obstructive pulmonary disease.

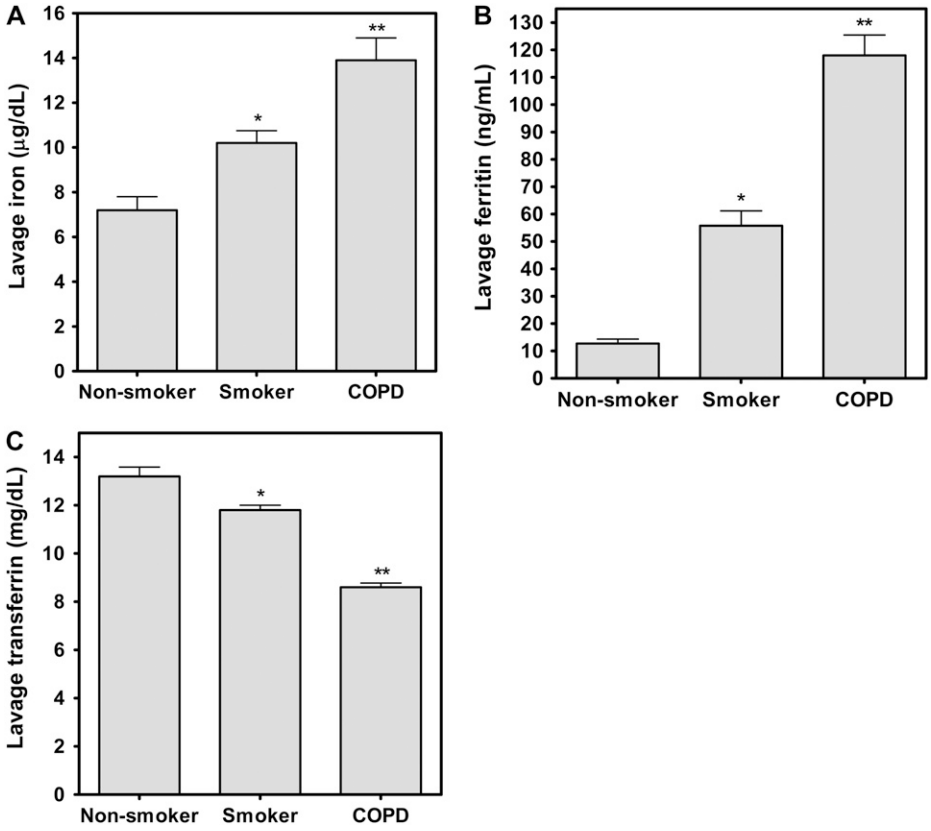

Figure 6. Lavage iron and ferritin concentrations increased, whereas transferrin concentrations decreased, in healthy smokers and patients with chronic obstructive pulmonary disease (COPD). Lavage iron (*A*) and ferritin (*B*) concentrations increased in healthy smokers, whereas transferrin concentrations (*C*) decreased. Patients with COPD, after smoking, had further elevations in iron (*A*) and ferritin (*B*), and decrements in transferrin (*C*). *Increased relative to nonsmoker; $P < 0.05$. **Significant differences relative to both nonsmokers and smokers; $P < 0.05$. Data presented are means ± SEM.

attributable to cigarette smoking itself. Therefore, changes of serum transferrin and iron in the rat exposed to cigarette smoke likely reflect limitations of an acute injury model in representing the human response to chronic smoking.

Tissue injuries after smoking are considered the product of oxidative stress. Indices of increased local and systemic oxidative stress are present in cigarette smokers (31–34). Lavage ascorbate concentrations were decreased in both the animal model with cigarette smoke exposure and in healthy cigarette smokers. Both the gas and particulate fractions of cigarette smoke are rich sources of radicals, but the former are short lived (32). Filtering the smoke eliminated this effect in animals, supporting an oxidative stress associated with particles in cigarette smoke. This oxidant generation by cells exposed to CSC was shown to be iron dependent. Cigarette smoke particles can be retained in the lungs of exsmokers and persist for the duration of life. During and after the mobilization of iron from host sources by a chelate other than a particle, this metal could catalyze radical production (35). Oxidant generation in cigarette smokers and exsmokers possibly results from a comparable catalysis by increased concentrations of metal resulting from complexation by the particle surface and its accumulation.

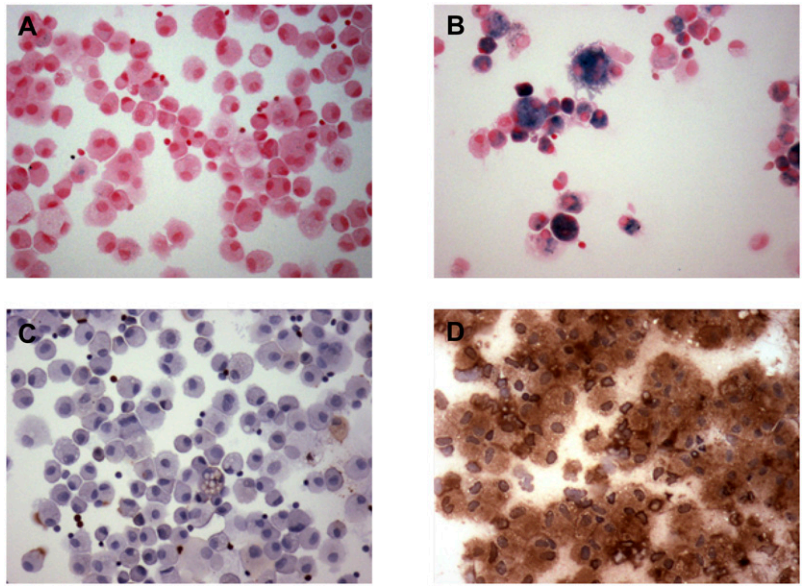

Figure 7. Iron and ferritin expression increased in lavage cytospins. Cells were pelleted onto slides and stained for iron and ferritin using a Perls' stain and immunohistochemistry, respectively. There was greater staining for metal in lavaged cells from healthy smokers (*B*) relative to those from healthy nonsmokers (*A*). Similarly, ferritin expression was increased among cells from smokers (*D*) relative to those from nonsmokers (*C*). Magnification, ~200×.

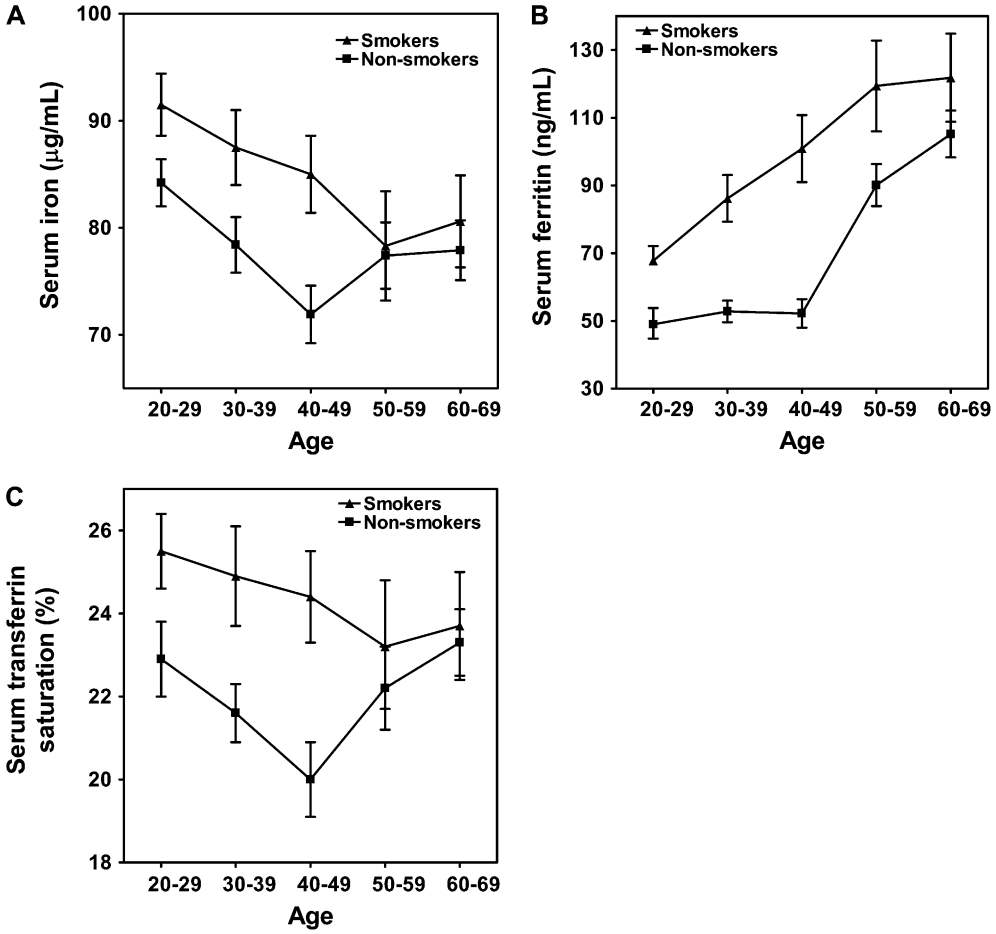

Figure 8. Serum indices of iron homeostasis revealed disparities between nonsmokers and smokers. Relative to nonsmokers, serum iron and ferritin concentrations and transferrin saturation in cigarette smokers were significantly increased. Analysis included 7,251 individuals aged 20 years and older, with information about age, race, sex, and smoking status available. Data presented are means ± SD.

Animals exposed to cigarette smoke displayed both increased lavage concentrations of an inflammatory mediator and a neutrophilic influx. Filtering with removal of the particles almost totally eliminated this effect of the cigarette smoke in animals. Lavage IL-8 was increased in healthy smokers and patients with COPD. At the cellular level, release of a pertinent inflammatory mediator after incubation with CSC was iron dependent. These data support some association between both metal accumulation by the particle, and oxidative stress with a proinflammatory event. With the accrual of a critical mass of particles in the human lung, cessation of smoking will not reverse alterations in iron homeostasis, oxidant generation, and inflammation. Retention of the particles in the lung, with the dependent oxidative stress and inflammation, can potentially cause COPD and cancer to develop in both current smokers and exsmokers.

We conclude that cigarette smoke particles alter iron homeostasis, and that this could contribute to disease after smoking. After exposure to cigarette smoke, elevations in catalytically active iron present an oxidative stress that triggers a cascade of biochemical events, culminating in inflammation. Particle retention in the lung will be associated with continued iron accumulation and possible injury. The metal can accumulate systemically, and this may increase the risk for other diseases (36, 37). Disparities in iron levels between the sexes may contribute to an increased risk for males in injuries associated with smoking (38). Therapies to prevent injury and disease after smoking could focus on cessation of the habit, minimizing particle retention in the lung, increasing particle clearance, and perhaps diminishing availability of iron in the host (39). Finally, a shared mechanism of biological activity could account for similarities in the clinical presentation after exposure to air pollution particles and cigarette smoking. An alteration in iron homeostasis with metal accumulation is proposed as contributing to both injuries (40).

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.

References

1. World Health Organization. The World Health report 2002: reducing risks, promoting healthy life. Geneva, Switzerland: World Health Organization; 2002.
2. Rutgers SR, Postma DS, ten Hacken NH, Kauffman HF, van Der Mark TW, Koeter GH, Timens W. Ongoing airway inflammation in patients with COPD who do not currently smoke. *Thorax* 2000;55:12–18.
3. Kabat GC. Aspects of the epidemiology of lung cancer in smokers and non-smokers in the United States. *Lung Cancer* 1996;15:1–20.
4. Johnson WR. Pyrogenesis and physiochemical nature of tobacco smoke. In: Tobacco smoke: its formation and composition. Kingsport, TN: Tennessee Eastman; 1977. pp. 1–26.
5. National Research Council. Environmental tobacco smoke: measuring exposures and assessing health effects. Washington, D.C.: National Academy Press; 1986.
6. Baker RR. Chapter 12: Smoke chemistry. In: Davis DL, Nielsen MT, editors. Tobacco-production, chemistry and technology. Oxford, UK: Blackwell Science; 2000. pp. 398–439.
7. Ghio AJ, Stonehuerner J, Pritchard RJ, Piantadosi CA, Quigley DR, Dreher KL, Costa DL. Humic-like substances in air pollution particulates correlate with concentrations of transition metals and oxidant generation. *Inhal Toxicol* 1996;8:479–494.
8. Ghio AJ, Stonehuerner J, Quigley DR. Humic-like substances in cigarette condensate and lung tissue of smokers. *Am J Physiol* 1994; 266:L382–L388.

9. Kragten J. Atlas of metal–ligand equilibria in aqueous solution. New York: Halstead Press; 1978.
10. Finelli VN, Petering HG. Effects of metal-binding fractions of tobacco smoke on *in vitro* activity of enzymes. *Arch Environ Health* 1972;25: 97–100.
11. Mussalo-Rauhamaa H, Leppanen A, Salmela SS, Pyysalo H. Cigarettes as a source of some trace and heavy metals and pesticides in man. *Arch Environ Health* 1986;41:49–55.
12. Breuer W, Epsztejn S, Cabantchik ZI. Iron acquired from transferrin by K562 cells is delivered into a cytoplasmic pool of chelatable iron(II). *J Biol Chem* 1995;270:24209–24215.
13. Smith KR, Veranth JM, Kodavanti UP, Aust AE, Pinkerton KE. Acute pulmonary and systemic effects of inhaled coal fly ash in rats: comparison to ambient environmental particles. *Toxicol Sci* 2006;93: 390–399.
14. van der Vaart H, Postma DS, Timens W, ten Hacken NH. Acute effects of cigarette smoke on inflammation and oxidative stress: a review. *Thorax* 2004;59:713–721.
15. Torrance JD, Bothwell TH. Tissue iron stores. In: Cook JD, editor. Iron. New York: Churchill Livingstone; 1980. pp. 90–115.
16. Ghio AJ, Turi JL, Madden MC, Dailey LA, Richards JD, Stonehuerner JG, Morgan DL, Singleton S, Garrick LM, Garrick MD. Lung injury after ozone exposure is iron-dependent. *Am J Physiol* 2007;292:L134–L143.
17. Kutnink MA, Skala JH, Sauberlich HE, Omaye ST. Simultaneous determination of ascorbic acid, isoascorbic acid (erythorbic acid), and uric acid in human plasma by high performance liquid chromatography with amperometric detection. *J Liq Chromatogr* 1985;8: 31–46.
18. Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol* 1985;113:548–555.
19. Roth MD, Connett JE, D'Armiento JM, Foronjy RF, Friedman PJ, Goldin JG, Louis TA, Mao JT, Muindi JR, O'Connor GT, *et al.*; FORTE Study Investigators. Feasibility of retinoids for the treatment of emphysema study. *Chest* 2006;130:1334–1345.
20. Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS, GOLD Scientific Committee. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 2001;163: 1257–1276.
21. Theil EC. Coordinating responses to iron and oxygen stress with DNA and mRNA promoters: the ferritin story. *Biometals* 2007;20: 513–521.
22. Wang X, Ghio AJ, Yang F, Dolan KG, Garrick MD, Piantidosi CA. Iron uptake and Nramp-2/DMT1/DCT1 in human bronchial epithelial cells. *Am J Physiol* 2002;282:L987–L995.
23. Quan SG, Golde DW. Identification and localization of toxic elements in normal human lung macrophages. *Proc Soc Exp Biol Med* 1981;167: 175–181.
24. Thompson AB, Bohling T, Heires A, Linder J, Rennard SI. Lower respiratory tract iron burden is increased in association with cigarette smoking. *J Lab Clin Med* 1991;117:493–499.
25. McGowan SE, Murray JJ, Parrish MG. Iron binding, internalization, and fate in human alveolar macrophages. *J Lab Clin Med* 1986;108:587–595.
26. McGowan SE, Henley SA. Iron and ferritin contents and distribution in human alveolar macrophages. *J Lab Clin Med* 1988;111:611–617.
27. Wesselius LJ, Nelson ME, Skikne BS. Increased release of ferritin and iron by iron-loaded alveolar macrophages in cigarette smokers. *Am J Respir Crit Care Med* 1994;150:690–695.
28. Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM. Ferritin: a cytoprotective antioxidant strategem of endothelium. *J Biol Chem* 1992;267:18148–18153.
29. El-Zayadi A-R. Heavy smoking and liver. *World J Gastroenterol* 2006; 12:6098–6101.
30. Avunduk AM, Yardimci S, Avunduk MC, Kurnaz L, Kockar MC. Determinations of some trace and heavy metals in rat lenses after tobacco smoke exposure and their relationships to lens injury. *Exp Eye Res* 1997;65:417–423.
31. Rahman I, MacNee W. Role of oxidants/antioxidants in smoking-induced lung diseases. *Free Radic Biol Med* 1996;21:669–681.
32. Pryor W. Cigarette smoke radicals and the role of free radicals in chemical carcinogenicity. *Environ Health Perspect* 1997;105:875–882.
33. Foronjy RF, Mirochnitchenko O, Propokenko O, Lemaitre V, Jia Y, Inouye M, Okada Y, D'Armiento JM. Superoxide dismutase expression attenuates cigaretee smoke- or elastase-generated emphysema in mice. *Am J Respir Crit Care Med* 2006;173:623–631.
34. MacNee W. Pulmonary and systemic oxidant/antioxidant imbalance in chronic obstructive pulmonary disease. *Proc Am Thorac Soc* 2005;2: 50–60.
35. Coffman TJ, Cox CD, Edeker BL, Britigan BE. Possible role of bacterial siderophores in inflammation: iron bound to the *Pseudomonas siderophore* pyochelin can function as a hydroxyl radical catalyst. *J Clin Invest* 1990;86:1030–1037.
36. Ford ES, Cogswell ME. Diabetes and serum ferritin concentration among US adults. *Diabetes Care* 1999;22:1978–1983.
37. Gajalakshmi V, Peto R, Kanaka TS, Jha P. Smoking and mortality from tuberculosis and other diseases in India: retrospective study of 43000 adult male deaths and 35000 controls. *Lancet* 2003;362:507–515.
38. Dransfield MT, Washko GR, Foreman MG, Estepar RS, Reilly J, Bailey WC. Gender differences in the severity of CT emphysema in COPD. *Chest* 2007;132:464–470.
39. Martinez JA, Guerra CC, Nery LE, Jardim JR. Iron stores and coagulation parameters in patients with hypoxemic polycythemia secondary to chronic obstructive pulmonary disease: the effect of phlebotomies. *Sao Paulo Med J* 1997;115:1395–1402.
40. Ghio AJ, Cohen MD. Disruption of iron homeostasis as a mechanism of biologic effect by ambient air pollution particles. *Inhal Toxicol* 2005; 17:709–716.