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Induction of Ferritin and Lipid Peroxidation by Coal Samples With Different Prevalence of Coal Workers' Pneumoconiosis: Role of Iron in the Coals

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Background *Differences in levels of bioavailable iron (BAI) in coal may be responsible for the observed regional differences in the prevalence and severity of coal workers' pneumoconiosis (CWP).*

Methods *Twenty-nine coal samples from three coal mine regions were tested in human lung epithelial Type II A549 cells. They were from Utah (UT), West Virginia (WV), and Pennsylvania (PA) with a prevalence of CWP of 4, 10, and 26%, respectively.*

Results *Low molecular weight (LMW) chelators bound iron, a fraction of BAI in the cells released from coals, ferritin, and lipid peroxidation were significantly higher in cells treated with various coals than in control cells, with an increasing order of UT < WV < PA, in parallel to the prevalence of CWP in these coal mine regions. Deferoxamine (DFO), a specific iron chelator, was used to distinguish effects of BAI from those of other transition metals. Our results indicate that BAI in the coals of WV and UT is the main metal species in inducing ferritin and lipid peroxidation. In contrast, biological effects of PA coals are not only from BAI, but from other transition metals as well.*

Conclusions *Based on a large number of coal samples from various seams, the findings of this study provide further evidence that metals, particularly iron, play important roles in coal dust-induced cellular damage, ultimately leading to the development of CWP and contributing to the regional differences in the prevalence of the disease.* Am. J. Ind. Med. 42:171–179, 2002. © *2002 Wiley-Liss, Inc.*

KEY WORDS: ferritin; iron; lipid peroxidation; coal workers' pneumoconiosis

INTRODUCTION

Nationwide, 55% of electricity is generated in coal-fired plants. With the current energy crisis in California, coal is looking more appealing than it has in years. Because the federal government has vowed to increase the exploration of coal and the coal industries are hiring more coal workers, a surge in ''black lung'' disease could be expected.

Three pathologies have been identified that are related to coal inhalation: pneumoconiosis including simple pneumoconiosis and progressive massive fibrosis (PMF), chronic obstructive bronchopneumopathy such as emphysema and/or chronic bronchitis, and stomach cancer [Ruckley et al., 1984; Miller and Jacobsen, 1985; Hurley et al., 1987; Morgan, 1999]. Among the respiratory diseases, coal workers' pneumoconiosis (CWP) has received the most attention because of its clear occupational association. Epidemiological studies of the relationship between the prevalence of CWP and environmental measurements have consistently revealed that the predominant adverse exposure factor is respirable mixed coal dusts [Attfield and Wagner, 1993].

Abbreviations: BAI, bio-available iron; CWP, coal workers' pneumoconiosis; DFO, deferoxamine; FBS, fetal bovine serum; LMW, low molecular weight; PMF, progressive massive fibrosis; RFU, relative fluorescence units.

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Despite comparable dust exposure levels, marked regional differences in the prevalence of CWP are well documented [Reisner and Robock, 1977; Hurley et al., 1982; Amoudru, 1987; Attfield and Castellan, 1992]. A decline in prevalence from East to West in the US, the disease being most common in the Pennsylvania (PA) coalminers and least common in miners from Utah (UT) [Morgan et al., 1973] has been reported. Although the disease is less prevalent now, mainly due to improved working conditions and dust control measures, the regional differences persist [Attfield and Seixas, 1995; De Vuyst and Camus, 2000; Meyer et al., 2001]. Coal rank is defined as the extent to which the organic materials are transformed over geological time from peat to anthracite; risk of CWP increases with coal rank [Miller and Jacobsen, 1985]. Coal rank is not an active compound, chemically speaking, which can induce biological responses. A correlation between coal rank and cell cytotoxicity has not been established in biological studies [Christian and Nelson, 1978; Christian et al., 1979].

The goal of the present study was to identify factors that contributed to the observed regional differences in the prevalence of CWP. CWP is considered as one of the human lung pathologies related to oxidative stress. We tested the role of bioavailable iron (BAI) in the coals in inducing oxidative cell damage, which may lead to the development of CWP. Human lung epithelial Type II A549 cells were treated with 29 coal samples from three coal mine regions with distinct differences in the prevalence of CWP. In mammalian cells, iron bound to low molecular weight (LMW) species, such as citrate, also termed ''LMW iron,'' is thought to be the active species responsible for generating oxidants [Jacobs, 1977; Toyokuni, 1996; Anderson, 1999]. If iron from coals becomes bioavailable in the cells, this fraction of iron should be first LMW bound. As a protective measure to withhold iron from oxidation-reduction (redox) reactions, ferritin, an iron storage protein, should be altered after the cells are treated with BAI-containing coals. As a consequence of redox reactions catalyzed by LMW iron in the A549 cells, levels of lipid peroxidation should also be altered. Therefore, increases in LMW iron, ferritin, and lipid peroxidation would prove our hypothesis that BAI in the coals became bioavailable in cells after phagocytosis. This would lead to the conclusion that different levels of BAI in the coals can trigger various degrees of cellular damage, which contributes to the regional differences in the prevalence and severity of CWP.

MATERIALS AND METHODS

Chemical Reagents

Sodium phosphate, potassium phosphate, bovine serum albumin (BSA), bicinchoninic acid kit, tetramethylbenzidine (TMB), glacial acetic acid, thiobarbituric acid (TBA), 10% SDS, butanol, calcein, deferoxamine mesylate (DFO), HEPES, alpha-Minimum Essential Medium (α-MEM) were purchased from Sigma Chemical Company (St. Louis, MO). Fetal bovine serum (FBS) and antibiotics such as antimycotic, and L-glutamine were obtained from Atlanta Biologicals (Atlanta, GA). Antibody to human spleen and liver ferritin were from Roche Molecular Biochemicals (Indianapolis, IN). EZ-link activated peroxidase was from Pierce (Rockford, IL). Ferritin was from Calbiochem (San Diego, CA). Gelatin and Tween-20 were from Bio-Rad (Richmond, CA). Ultrafree-MC filter units (10,000 NMWL) were from Millipore (Bedford, MA). Twenty-nine coal samples from three coal mine regions of UT, West Virginia (WV), and PA were purchased from the Penn State Coal Sample Bank (University Park, PA, http://www.energyinstitute.psu.edu/copl/doesb.htm).

Preparation of Respirable Size Coal Dusts for Cell Treatments Using a Mercer Impactor

Coal samples were ground and size-classified using the Mercer Impactor (Intox, Albuquerque, NM). In brief, coal samples were ground in a ball mill with 5-mm diameter glass beads for 24 hr. An aliquot (30 g) of the ground coal sample was resuspended in a 1-L glass jar with 5 L of nitrogen (N_2) per minute. Coal dusts larger than 10 μm were removed by passing them through a cyclone (BGI, Waltham, MA) with a 10-μm cut-off size, and the respirable fraction of the coal dusts were collected on Teflon filters (0.45 μm pore size, Millipore). The size distribution of the coal dusts collected on the Teflon filter was determined using the Mercer Impactor. The diameters of coal particles collected for cell treatment were less than 5 μm. They were stored in a dessicator at 4°C to inhibit microbial growth. Particles were suspended in culture medium (1 mg/ml) without FBS and briefly sonicated in an ultrasonic water bath (Branson, CT) before use.

Cell Culture and Treatments

Human lung epithelial cell line, A549, with characteristics of alveolar epithelial Type II cells, was obtained from American Type Culture Collection (ATCC CCL185, Bethesda, MD). Cells were maintained in α-MEM completed with 10% FBS, 1% antibiotics, and 1% L-glutamine and grown in 5% CO_2 at 37°C.

A549 cells were plated in 35-mm 6-well culture dishes (surface area/well about 10 cm^2). At 80–90% confluence, cells were exposed to different doses of coal particles (0, 1, 2, 5, 10 $\mu g/cm^2$) for various times with or without FBS. Exposure to coal dusts was expressed in microgram per square centimeter, because at these exposure conditions the dusts were not completely soluble. In other experiments, coal suspensions were treated with DFO (0.5 mM), an iron

chelator, for 30 min before adding them to the cells. Cells without the addition of coal suspensions or DFO were used as controls.

Ferritin Measurements in A549 Cells Treated With Coal Dusts

After exposure to various coal dusts, cells in 6-well culture dishes were washed twice with ice-cold phosphate-buffered saline (PBS), then harvested by gentle scraping with a rubber policeman. Cells were finally suspended in cold distilled water at a concentration of 10^6/ml, and lysed by freeze-thawing in liquid nitrogen and in a water bath at 37°C. After centrifugation at 9,000*g* at 4°C for 20 min, the supernatants were stored at −80°C for the ferritin assay. Levels of ferritin in A549 cell lysates were determined according to a previously published protocol [Fang and Aust, 1997]. In brief, an antibody to a mixture of human spleen and liver ferritin was used as the capture antibody to coat an ELISA plate. Human liver ferritin was used as the standard. The conjugate of peroxidase and antibody to human spleen and liver ferritin was then added to serve as the detector to determine the amount of ferritin bound to the capture antibody. TMB was then added as the substrate for the peroxidase, and the absorbance of the oxidation product of TMB was determined at 450 nm using a microplate reader (SpectroMax Plus, Molecular Devices, Sunnyvale, CA). Total protein in the cell lysates was determined using bicinchoninic acid, and the results were expressed as microgram of ferritin per milligram protein.

Measurements of Lipid Peroxidation

After exposure to various coal dusts, A549 cells in 6-well culture dishes were washed twice with ice cold PBS to remove coal dusts. Cells were then lysed in each well using 1 ml cell lysis buffer (10 mM HEPES, pH 8.0, 0.2% SDS). Cell lysates were collected in 1.5 ml Eppendorf tubes, and centrifuged at 2,500*g* for 20 min. One part of the supernatants was used for lipid peroxidation assay, while the other for determination of LMW iron (see below). Lipid peroxides in the coal dust treated cells were quantitated by TBA assay [Huang et al., 1994]. Cell lysates (0.4 ml) were mixed with an equal volume of 0.4% TBA in 10% acetic acid, pH 5.0, then heated to 90°C for 1 hr. After cooling under cold tap water, 0.8 ml of butanol was added and the mixtures were vigorously shaken. After centrifugation at 2,500*g* for 10 min, the fluorescence intensities of TBA reactive substances in the butanol phase were measured using a fluorescence-chemiluminescence Microplate reader (Gemini, Molecular Devices) at an excitation wavelength of 515 nm (bandwidth 4.5 nm), and an emission wavelength of 553 nm (bandwidth 9.0 nm). The results for lipid peroxidation were expressed as relative fluorescence units (RFU) per milligram protein.

Measurements of LMW Iron in Cell Lysates

LMW iron was measured as follows: 200 μl of the cell lysates were centrifuged through a 10 kDa Ultra-free filter unit (10,000*g* at 4°C for 30 min). LMW iron in the cells was determined by calcein, a fluorescent iron chemosensor [Cabantchik et al., 1996; Epsztejn et al., 1997; Ali et al., 2002]. Calcein binds iron stoichiometrically, with its fluorescence quenched upon calcein-Fe complex formation. By adding DFO, a strong iron chelator, the fluorescence specifically quenched by iron can be regenerated. Therefore, the differences in fluorescence readings of the same sample with or without addition of DFO are specifically proportional to the amounts of iron. In brief, each well of the microtiter plate contained 80 μl calcein (5 μM), 20 μl HEPES (500 mM), pH 7.4, and 20 μl distilled H_2O. Samples or iron standards (80 μl) were then added to the wells. The plate was covered and incubated in a water bath at 37°C for 20 min. The fluorescence was then determined using a fluorescence-chemiluminescence microplate reader at an excitation at 485 nm (bandwidth 4.5 nm), and an emission at 515 nm (bandwidth 9 nm). To reverse iron-induced calcein fluorescence quenching, 5 μl DFO (1 mM) were added to the samples. The reaction mixtures were incubated in a water bath at 37°C for 10 min. The net fluorescence quenched by iron was calculated from the difference in fluorescence measurements of the same sample with or without (±) DFO. The differences in FRU between ± DFO versus $FeSO_4$ standards were used to construct a standard curve for calculating levels of the LMW-iron in the cell lysates. The results for LMW iron were expressed as nanomoles of iron per milligram protein.

Data Analysis

The experimental results were analyzed for their statistical significance by the paired, two-tailed Student's *t*-test. A confidence level of $P < 0.05$ was taken to represent a significant difference between the two means.

RESULTS

Effect of Coal Dusts on the Induction of Ferritin in A549 Cells

Ferritin levels in A549 cells increased with increasing coal dust concentration from 1 to 10 μg/cm^2 (Table I). Among the three coal samples tested, the coal (Psoc 1198) from the PA coal mine region was the most active in inducing ferritin in A549 cells, followed by the coals from WV and UT coal mine regions. Cells treated with the PA coal showed a time- and dose-dependent increase in ferritin. Levels of ferritin were significantly higher in cells treated with the PA coal than

TABLE I. Induction of Ferritin in Human Lung Epithelial A549 Cells by Three Coal Samples Each From PA, WV, and UT Coal Mine Regions

	1.0 μg/cm^2	2.0 μg/cm^2	5.0 μg/cm^2	10.0 μg/cm^2
24 hr				
UT (Psoc 459)	0.13 ± 0.01	0.15 ± 0.003	0.23 ± 0.01[a]	0.43 ± 0.05[a]
WV (Psoc 1519)	0.14 ± 0.01	0.19 ± 0.01	0.34 ± 0.04[a]	0.40 ± 0.03[a]
PA (Psoc 1198)	0.18 ± 0.02	0.40 ± 0.01[a]	0.57 ± 0.01[a,b,c]	0.65 ± 0.03[a,b,c]
48 hr				
UT (Psoc 459)	0.15 ± 0.02	0.14 ± 0.01	0.20 ± 0.01[a]	0.32 ± 0.02[a]
WV (Psoc 1519)	0.14 ± 0.01	0.23 ± 0.03	0.33 ± 0.02[a,b]	0.43 ± 0.01[a,b]
PA (Psoc 1198)	0.32 ± 0.05[a]	0.56 ± 0.07[a]	0.76 ± 0.09[a,b,c]	1.07 ± 0.08[a,b,c]

[a] $P < 0.05$, as compared to controls. A549 cells were treated with three coals in the presence of 10% FBS. Average ferritin levels in the control cells were 0.17 ± 0.01 μg/mg protein. Ferritin levels in the control cells treated for 24 and 48 hr were similar and were combined for statistical analyses. Results are expressed as mean ± Standard Error (SE) of three independent experiments, each in duplicates.
[b] $P < 0.05$, as compared to UT.
[c] $P < 0.05$, as compared to WV.

in the control cells, and in the cells treated with the coals from WV and UT at 5 μg/cm^2 or higher. In cells treated with the WV coal (Psoc-1519), levels of ferritin also increased as a function of dose, but independent of the duration of treatment. Levels of ferritin in cells treated with the UT coal (Psoc 459) were significantly increased in comparison with the untreated cells, only at a dose of 5 μg/cm^2 or higher. However, the increased levels in ferritin declined when the treatment was extended to 48 hr. This was in contrast with the no change for the WV coal and an increase from 24 to 48 hr treatment for the PA coal.

LMW Iron Detection in Coal Dust-Treated A549 Cells

Table II shows that coals from UT, WV, and PA provided release of increasing levels of LMW iron in A549 cells, which parallel the prevalence of 4, 10, and 26% CWP from the corresponding coal mine regions, respectively. At 5 μg/cm^2, 48 hr treatment, LMW iron concentration in the cell lysates reached 0.54 ± 0.037 μM in PA coal-treated cells and 0.42 ± 0.015 μM in WV coal-treated cells (Table II). LMW iron concentrations in the lysates of the UT coal-treated cells were close to the controls at 0.35 ± 0.013 μM. However, when data were expressed as nanomoles of iron per milligram protein in the cell lysates, differences in LMW iron levels became greater and statistically significant. The highest LMW iron levels occurred in cells treated with the PA coal, followed by the coals from WV and UT (Table II). These changes were likely due to the slight cytotoxicity induced by the coal samples (48 hr treatment), which resulted in lower cell numbers and less protein in the lysates. Using Trypan blue exclusion assay, the cell viability was approximately 85% in the cells treated with the three coals. Interestingly, levels of LMW iron as nanomole iron per milligram protein were positively correlated with levels of ferritin shown in the Table I (correlation coefficient r = 0.96, 5 μg/cm^2 for 48 hr) (data not shown). These results suggest that LMW iron released from coal may be responsible for the induction of ferritin in A549 cells.

TABLE II. Levels of LMW Iron in A549 Cells Treated With Three Coal Samples Each From PA, WV, and UT Coal Mine Regions*

	LMW iron μM	LMW iron nmoles/mg protein
Control	0.36 ± 0.009	1.56 ± 0.11
UT (Psoc 459)	0.35 ± 0.013	1.89 ± 0.085[a]
WV (Psoc 1519)	0.42 ± 0.015[a,b]	2.43 ± 0.25[a]
PA (Psoc 1198)	0.54 ± 0.037[a,b,c]	3.18 ± 0.17[a,b,c]

*In the presence of 10% FBS, A549 cells were treated with three coals at 5 μg/cm^2 for 48 hr. Levels of LMW iron in the cell lysates were determined by fluorescent calcein, as described in Methods. Data are expressed as micromolar in the lysates or nanomole Fe per milligram protein. Results are presented as mean ± SE of three independent experiments.
[a] $P < 0.05$, as compared to control.
[b] $P < 0.05$, as compared to UT.
[c] $P < 0.05$, as compared to WV.

Effects of DFO on Ferritin Induction and Lipid Peroxidation in A549 Cells Treated With Coals

To further confirm the hypotheses that LMW iron released from coals induces ferritin as well as lipid peroxidation, we used DFO, an iron chelator, to prevent LMW iron from becoming bioavailable. Coal samples were suspended in 0.5 mM DFO solution before adding them to cells. Figure 1 shows that the pretreatment of aqueous coal suspensions with DFO significantly decreases levels of

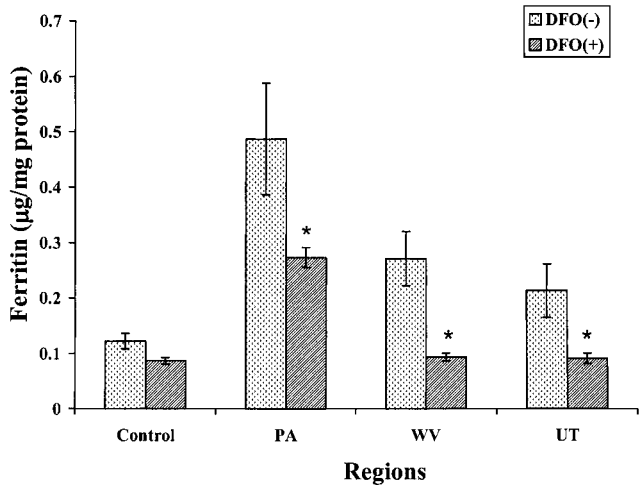

FIGURE 1. Effects of DFO on ferritin induction by various coal samples in A549 cells. Coals from PA (PSOC 1198), WV (PSOC 1519), and UT (PSOC 459) were suspended in α-MEM media containing 0.5 mM DFO for 30 min before adding them to cells. In the presence of 10% FBS, cells were treated with coals at 10 $\mu g/cm^2 \pm$ DFO for 24 hr. Results are expressed as means $\pm$ standard error (SE) of three independent experiments (n = 3). *Significantly different from the coals without DFO pretreatment ($P < 0.05$) by Student's *t*-test.

ferritin in A549 cells as compared to the coal samples without DFO. Addition of DFO (final concentration 45.5 μM in tissue culture media) to the control cells without coal treatment also leads to a decrease in ferritin. It is noteworthy that the significant increases in ferritin in cells treated with coals from WV and UT were completely attenuated by DFO, levels of ferritin similar to those of the control cells. In contrast, DFO pretreatment could not fully inhibit the increase in ferritin in cells treated with the PA coal, though we have calculated that 0.5 mM DFO is sufficient to chelate all iron released by the PA coal. It remained significantly higher than the controls.

Similar to ferritin induction, DFO pretreatment decreased lipid peroxidation in all samples tested, being statistically significant in cells treated with the WV and UT coals (Fig. 2). In cells treated with the PA and WV coals, DFO pretreatment did not completely prevent cells from lipid peroxidation. Levels of lipid peroxidation in those cells were significantly higher than in the control cells with or without DFO pretreatment. In an aqueous solution mimicking the conditions of phagolysosomes of cells (10 mM phosphate, pH 4.5), we found that the coals from the PA coal mine region were able to release not only iron (average level of Fe^{2+}: 7288.2 ppm, w/w, from eight coal samples) but copper (0.32 ppm), nickel (0.4 ppm), and other transition metals as well (e.g., As, 0.13 ppm) [Zhang et al., 2002]. These data suggest that besides LMW iron, other transition metals also contribute to the coal dust-induced lipid peroxidation.

Regional Differences in LMW Iron, Ferritin, and Lipid Peroxidation

Table III provides a summary of LMW iron, ferritin, and lipid peroxidation in A549 cells treated with 29 coal samples from three coal mine regions. As mentioned before, prevalence of pneumoconiosis for PA, WV, and UT coal workers was 26, 10, and 4%, respectively. We have shown in Table III that average levels of LMW iron, ferritin, and lipid peroxidation were the highest in cells treated with coals from PA (n = 8), intermediate in cells treated with coals from WV (n = 10), and lowest in UT coal treated cells (n = 11).

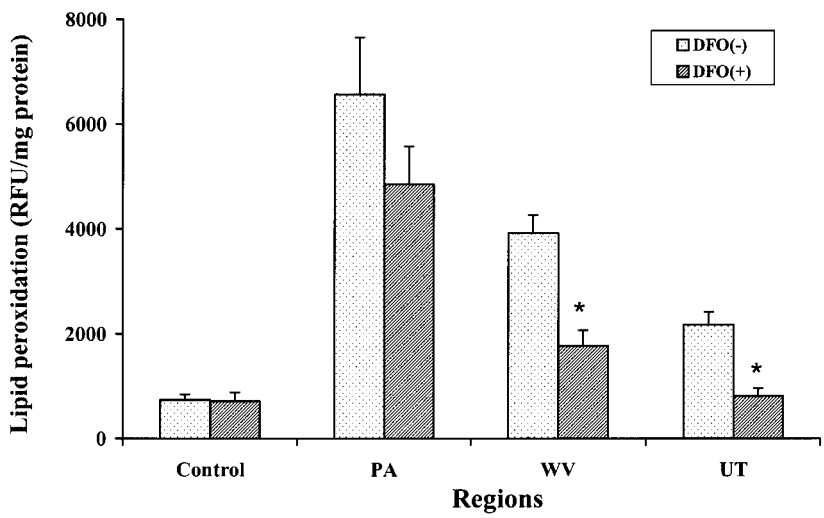

FIGURE 2. Effects of DFO on lipid peroxidation induced by various coal samples in A549 cells. The same coals as in Figure 1 were suspended in α-MEM medium containing 0.5 mM DFO for 30 min before treatment. Coals without DFO pretreatment were used for comparison. Results are expressed as means $\pm$ SE of three independent experiments (n = 3). *Significantly different from the coals without DFO pretreatment ($P < 0.05$) by Student's *t*-test.

TABLE III. Summary of Ferritin, LMW Iron, and Lipid Peroxide Formation in A549 Cells Treated With 29 Coal Samples From the PA, WV, and UT Coal Mine Regions*

Coal region	Sample no.	LMW iron	Ferritin	Lipid peroxidation
Control	1	1.11	0.15	4564.9
	2	1.56	0.34	3537.4
	3	1.49	0.25	5143.6
	4	1.46	0.25	4568.6
	5	1.68	0.25	2461.7
	6	1.10	0.26	4738.5
Mean ± SE		1.37 ± 0.11	0.25 ± 0.03	4169.1 ± 404.3
UT (4% CWP#)	313	2.05	0.63	4324.6
	498	4.58	0.22	6000.9
	462	3.04	0.25	5576.4
	500	1.01	0.23	3884.5
	1502	3.24	0.17	2444.3
	433	2.40	0.28	6413.9
	432	2.91	0.61	6421.8
	431	3.14	0.72	6044.9
	429	3.04	0.2	7412.4
	459	1.72	0.55	4642.1
	1112	2.35	0.33	4186.5
Mean ± SE		2.68 ± 0.28[a]	0.38 ± 0.06[a]	5213.9 ± 434.7[a]
WV (10% CWP)	731	1.05	0.26	4442.2
	130	1.86	0.31	5435.3
	702	6.71	2.31	7661.9
	816	5.91	1.19	9224.5
	1508	2.69	0.20	3055.2
	1519	4.81	0.87	3664.5
	727	1.62	0.25	5170.3
	895	1.49	0.24	4541.3
	896	3.95	0.40	6632.9
	997	3.52	1.04	5833.1
Mean ± SE		3.36 ± 0.62[a]	0.71 ± 0.21[a]	5566.1 ± 590.6[a]
PA (26% CWP)	260	1.66	0.28	6663.4
	324	5.06	1.06	7368.8
	325	8.27	0.84	7686.1
	337	6.30	1.22	9190.3
	1197	2.34	0.34	12467.9
	1198	6.56	2.04	6867.1
	1313	8.32	0.56	13217.4
	1516	3.90	0.76	8311.6
Mean ± SE		5.30 ± 0.89[a,b,c]	0.89 ± 0.21[a,b]	8971.6 ± 893.6[a,b,c]

#Prevalence of CWP was from Morgan et al., 1973.

*A549 cells were first cultured in complete α-MEM in 35 mm, 6-well culture dishes, and then serum starved overnight. Twenty-nine coal samples from three coal mine regions of PA, WV, and UT were size classified, and suspended in serum-free α-MEM immediately before use. Cells were treated at a single dose of 10 μg/cm^2 with each of 29 coal samples for 24 hr. Ferritin was determined by ELISA and LMW iron and lipid peroxidation were determined by fluorescence assays as described in Methods. Cells not treated with a coal sample were used as controls and shown for comparison. Data are expressed as nanomole, milligram, and relative fluorescence units (RFU) per milligram protein for LMW iron, ferritin, and lipid peroxidation, respectively. Results for each region are averaged and presented as mean ± SE of the coal samples from that region.

[a] $P < 0.05$, as compared to control.

[b] $P < 0.05$, as compared to UT.

[c] $P < 0.05$, as compared to WV.

Interestingly, prevalence of CWP is positively correlated with the average levels of LMW iron (correlation coefficient $r = 0.98$, $n = 4$), ferritin ($r = 0.94$), and lipid peroxidation ($r = 0.99$) from the corresponding coal mine region. Based on the 35 measurements (29 coals plus 6 controls), we determined that levels of LMW iron correlated with levels of ferritin (correlation coefficient $r = 0.69$, $n = 35$) and lipid peroxidation ($r = 0.60$).

DISCUSSION

CWP is one of the best-documented diseases in the field of occupational epidemiological studies. Many confounding factors such as dust concentration, years of underground exposure, job title, mining techniques, X-ray reader variation, as well as age and smoking status were taken into account in these studies. It has been shown in the US, Great Britain, France, and Germany that the prevalence and severity of CWP varied markedly among different coal mines, which could not be explained by the above cited confounding factors [Reisner and Robock, 1977; Hurley et al., 1982; Amoudru, 1987; Attfield and Castellan, 1992]. For example, the first round of the US National Study of CWP (NSCWP), which was completed in 1971, has examined a total of 9,076 miners from 29 bituminous and 2 anthracite mines [Morgan et al., 1973]. During that period, the average exposure in these mines was 3 mg/m^3. It was found that 41% of the eastern PA anthracite miners had simple pneumoconiosis and a further 14% had PMF (a total of 55% CWP). Meanwhile, 21% of the eastern PA bituminous coalminers had simple pneumoconiosis and 5% had PMF, but the comparable figures for bituminous miners in Colorado and UT were 4 and 0.4%.

In the present study, we have postulated that levels of BAI in the coals may be different among regions, which may cause the observed regional difference in prevalence and severity of CWP. Because of the available information in the first round of NSCWP, we were able to identify the coal seams, county, and states where the epidemiological studies were done. The Penn State Coal Sample Bank currently contains over 1400 well-documented coal samples that were collected nationwide since 1967. These coal samples are well characterized, relatively representative of the coal seam, and have been preserved under nitrogen. An array of data (geographic, sampling details, chemical, petrographic, mineralogical, etc.) can be provided for any coal requested. This allowed us to use the representative coal samples collected at the time when the first round of NSCWP was performed. This also made testing our hypothesis possible by correlating the physico-chemical parameters that we thought important in coal's toxicity with the prevalence of CWP.

Selection of coal samples in the present study was based on the first round of NSCWP. A total of 29 coal samples were from three coal mine regions of different seams or different locations at the same seam. They were well homogenized and characterized according to the American Standards for Testing and Materials (ASTM) methods. Upon receipt in our laboratory, coal particles were size-classified (≤ 5 μm) (data not shown). The A549 cell line is the most widely used cell line in studying diseases and mechanisms related to inhalation of particles. It has been shown that the fibrous particles, crocidolite asbestos, and coal fly ash produce ROS and induce ferritin in A549 cells [Chao et al., 1994; Park and Aust, 1998; Smith et al., 2000; Prahalad et al., 2001]. In the present study, we found that all coal samples significantly increased levels of LMW iron, ferritin, and lipid peroxidation in A549 cells. The coals from UT, which had the least BAI among the 29 samples tested [Huang et al., 1998; Zhang et al., 2002], triggered the lowest biological responses but were statistically different from the controls. Interestingly, the average levels of LMW iron, ferritin, and lipid peroxidation induced by the coal samples from each region correlated well with the prevalence of CWP, indicating that BAI in the coals contributes to the regional differences in the prevalence and severity of CWP.

To further confirm this hypothesis, DFO was used in an aqueous coal suspension before cell treatments. DFO can bind Fe^{3+}, as well as Al^{3+} and Ga^{3+}. However, neither Al^{3+} nor Ga^{3+} ions are likely to be found in aqueous coal suspensions at high concentrations. Al and Ga are trace metals in the ppm or ppb range according to the data provided by the Penn State Coal Sample Bank. DFO can inhibit the redox reaction of Fe^{2+} because it shifts the balance of equation from Fe^{2+} to Fe^{3+} and binds Fe^{3+} tightly. Therefore, the biological responses inhibited by the pretreatment with DFO are solely due to the presence of iron. It is noteworthy that ferritin induction by the coals from WV and UT was completely abolished by the pretreatment of coals with DFO (Fig. 1). Pretreatment of the PA coal with DFO showed a 44% inhibition in ferritin induction. Levels of ferritin in those cells remained significantly higher than the control levels. Similarly, DFO pretreatment with the aqueous coal suspensions inhibited lipid peroxidation induced by the coals from UT and WV, but not from PA (Fig. 2). These results suggest that transition metals other than BAI may participate in the induction of ferritin and lipid peroxidation in the PA coal-treated samples.

Using a large number of coal samples and testing them in an in vitro system, our study showed the homogeneity and heterogeneity of coal samples from various seams of the same coal mine region. It can be seen from Table III that there are variations in the levels of LMW iron, ferritin, and lipid peroxidation from different coal samples of the same region (e.g., UT). However, the intra-regional variation is small compared with the differences among regions. This is consistent with our previous observation on the buffering capacity and acid soluble Fe^{2+} content in the US coals measured in a cell-free system [Huang et al., 1998].

Regarding heterogeneity of the coal samples, treatments of the A549 cells with the three coal samples of PA (Psoc 1198), WV (Psoc 1519), and UT (Psoc 459) showed regional differences in repeated measurements of lipid peroxidation and ferritin induction (Figs. 1 and 2). However, this trend was not observed in levels of lipid peroxidation induced by the same WV and UT coals in Table III with one single measurement. We have also shown a positive correlation between acid-soluble Fe^{2+} content in five French coals and prevalence of CWP in coal miners working in the coal mine regions where the coal samples were collected (correlation coefficient $r = 0.94$) (Huang et al., 1999). In contrast, the present study tested 29 samples in the live A549 cells, which can provide a much more complex and efficient clearance system than the acidic media that we have been using [Huang et al., 1998, 1999]. We believe that the correlation between levels of LMW iron, ferritin, and lipid peroxidation in A549 cells and prevalence of CWP is not a coincidence, but probably a cause-effect relationship. As shown by the present study, BAI, as well as other bioavailable transition metals are likely to be the active compounds in inducing oxidative cell damage, which ultimately leads to the development of CWP. To support these observations, levels of ferritin and LMW iron were lower in coal-treated cells in the presence of 10% FBS (Tables I and II) as compared to coal-treated cells in the absence of FBS (Table III). These results suggest that transferrin present in FBS can chelate some BAI released from the coal samples, and thus protect cells from the induction of ferritin and lipid peroxidation. Therefore, the differences in the BAI and other bioavailable transition metals in the coals may be responsible for the regional differences in the prevalence and severity of CWP. Moreover, our study has shown that ferritin is sensitive to the presence of BAI and other transition metals in the coals. Because levels of coal dusts in the lung are difficult to quantitate, changes in levels of ferritin in sera of coal miners resulting from the alterations in coal dust inhalation may be used as a biomarker of exposure to coal dust.

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REFERENCES

Ali AM, Zhang Q, Dai J, Huang X. 2002. Calcein as a fluorescent iron chemosensor for the determination of low molecular weight iron in biological fluids. BioMetals (in press).

Amoudru C. 1987. Pneumoconiosis—actual dimensions of the problem. In: Le Bouffant L, editor. Silicosis and mixed-dusts pneumoconiosis. Paris: Colloque INSERM 155:3–40.

Anderson GJ. 1999. Non-transferrin-bound iron and cellular toxicity. J Gastroenterol Hepatol 14:105–108.

Attfield MD, Castellan RM. 1992. Epidemiological data on US coal miners' pneumoconiosis, 1960 to 1988. Am J Public Health 82:964–970.

Attfield MD, Seixas NS. 1995. Prevalence of pneumoconiosis and its relationship to dust exposure in a cohort of US bituminous coal miners and ex-miners. Am J Ind Med 27:137–151.

Attfield M, Wagner G. 1993. Respiratory disease in coal miners. In: Rom WN. editor. Environmental and occupational medicine. 2nd edn. Boston: Little, Brown, and Company. p 325–344.

Cabantchik ZI, Glickstein H, Milgram P, Breuer W. 1996. A fluorescence assay for assessing chelation of intracellular iron in a membrane model system and in mammalian cells. Anal Biochem 233:221–227.

Chao CC, Lund LG, Zinn KR, Aust AE. 1994. Iron mobilization from crocidolite asbestos by human lung carcinoma cells. Arch Biochem Biophys 314:384–391.

Christian RT, Nelson J. 1978. Coal: Response of cultured mammalian cells corresponds to the prevalence of coal workers pneumoconiosis. Environ Res 15:232–241.

Christian RT, Nelson JB, Cody TE, Larson E, Bingham E. 1979. Coal workers' pneumoconiosis: In vitro study of the chemical composition and particle size as causes of the toxic effects of coal. Environ Res 20:358–365.

De Vuyst P, Camus P. 2000. The past and present of pneumoconioses. Curr Opin Pulm Med 6:151–156.

Epsztejn S, Kakhlon O, Glickstein H, Breuer W, Cabantchik I. 1997. Fluorescence analysis of the labile iron pool of mammalian cells. Anal Biochem 248:31–40.

Fang R, Aust AE. 1997. Induction of ferritin synthesis in human lung epithelial cells treated with crocidolite asbestos. Arch Biochem Biophys 340:369–375.

Huang X, Zhuang Z, Frenkel K, Klein CB, Costa M. 1994. The role of nickel and nickel-mediated reactive oxygen species in the mechanism of nickel carcinogenesis. Environ Health Perspect 3(102 Suppl):281–284.

Huang X, Fournier J, Koenig K, Chen LC. 1998. Buffering capacity of coal and its acid-soluble Fe^{2+} content: Possible role in coal workers' pneumoconiosis. Chem Res Toxicol 11:722–729.

Huang X, Zalma R, Pezerat H. 1999. Chemical reactivity of the carbon-centered free radicals and ferrous iron in coals: Role of bioavailable Fe^{2+} in coal workers pneumoconiosis. Free Radic Res 30:439–451.

Hurley JF, Burns J, Copland L, Dodgson J, Jacobsen M. 1982. Coalworkers' simple pneumoconiosis and exposure to dust at 10 British coalmines. Br J Ind Med 39:120–127.

Hurley JF, Alexander WP, Hazledine DJ, Jacobsen M, Maclaren WM. 1987. Exposure to respirable coalmine dust and incidence of progressive massive fibrosis. Br J Ind Med 44:661–672.

Jacobs A. 1977. Low molecular weight intracellular iron transport compounds. Blood 50:433–439.

Meyer JD, Holt DL, Chen Y, Cherry NM, McDonald JC. 2001. SWORD '99: Surveillance of work-related and occupational respiratory disease in the UK. Occup Med (Lond) 51:204–208.

Miller BG, Jacobsen M. 1985. Dust exposure, pneumoconiosis, and mortality of coalminers. Br J Ind Med 42:723–733.

Morgan WK. 1999. Coal mining and COPD. Thorax 54:187.

Morgan WKC, Burgess DB, Jacobson G, O'Brien RJ, Pendergrass E, Reger RB, Shoub EP. 1973. The prevalence of coal workers' pneumoconiosis in US coal miners. Arch Environ Health 27:221–230.

Park SH, Aust AE. 1998. Regulation of nitric oxide synthase induction by iron and glutathione in asbestos-treated human lung epithelial cells. Arch Biochem Biophys 360:47–52.

Prahalad AK, Inmon J, Dailey LA, Madden MC, Ghio AJ, Gallagher JE. 2001. Air pollution particles mediated oxidative DNA base damage in a cell free system and in human airway epithelial cells in relation to particulate metal content and bioreactivity. Chem Res Toxicol 14:879–887.

Reisner MTR, Robock K. 1977. Results of epidemiological, mineralogical and cytotoxicological studies on the pathogenicity of coal mine dusts. Oxford, UK: Pergamon Press. p 703–715.

Ruckley VA, Gauld SJ, Chapman JS, Davis JM, Douglas AN, Fernie JM, Jacobsen M, Lamb D. 1984. Emphysema and dust exposure in a group of coal workers. Am Rev Respir Dis 129:528–532.

Smith KR, Veranth JM, Hu AA, Lighty JS, Aust AE. 2000. Interleukin-8 levels in human lung epithelial cells are increased in response to coal fly ash and vary with the bioavailability of iron, as a function of particle size and source of coal. Chem Res Toxicol 13:118–125.

Toyokuni S. 1996. Iron-induced carcinogenesis: The role of redox regulation. Free Radic Biol Med 20:553–566.

Zhang Q, Dai JS, Ali AM, Chen LC, Huang X. 2002. Roles of bioavailable iron and calcium in coal dust-induced oxidative stress: Possible implications in coal workers' lung disease. Free Radic Res 36:285–294.