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Roles of Bioavailable Iron and Calcium in Coal Dust-induced Oxidative Stress: Possible Implications in Coal Workers' Lung Disease

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Marked regional differences in prevalence of pneumoconiosis are apparent in the US despite comparable dust exposure. In the present study, we examined the ability of 28 coal samples to release bioavailable iron (BAI) and calcium, as well as other metals such as Cr, Ni, Cu, and Co, from three coalmine regions in Utah (UT), West Virginia (WV), and Pennsylvania (PA), respectively. BAI is defined as iron (both Fe^{2+} and Fe^{3+}) released by the coals in 10 mM phosphate solution, pH 4.5, which mimics conditions of the phagolysosomes in cells. We found that coals from the UT, WV, and PA regions released average levels of BAI of 9.6, 4658.8, and 12149 parts per million (ppm, w/w), respectively, which correlated well with the prevalence of pneumoconiosis from that region (correlation coefficient $r = 0.92$). The low levels of BAI in the UT coals were due to the presence of calcite ($CaCO_3$), which was shown to be preferentially acid solubilized before iron compounds. Release of iron by two coal samples from the PA and UT regions was further examined *in vitro* in human lung epithelial A549 cells. We found that the coal from PA, with a high prevalence of pneumoconiosis, released BAI in a dose-dependent manner, both in tissue culture media and in A549 cells. At 2 $\mu g/cm^2$, levels of lipid peroxidation induced by the PA coal were increased 112% over control cells at 24 h treatment, and were sustained at this level for 3 days. The coal from UT, with a low prevalence of pneumoconiosis, induced a marginal increase in cellular iron at 5 and 10 $\mu g/cm^2$ treatments and had no effect on lipid peroxidation. Calcium levels in the cells treated with the PA and UT coals were 8.6 and 11.5 $\mu moles/10^6$ cells, respectively, and were significantly higher than that in the controls (53 $\mu moles/10^6$ cells). Our results suggest that the differences in the BAI content in the coals may be responsible for the observed regional differences in the prevalence of pneumoconiosis. Therefore, BAI may be a useful characteristic of coal for predicting coal's toxicity.

Keywords: Coal; Pneumoconiosis; Chronic obstructive pulmonary disease; Oxidative stress; Iron; Calcium

Abbreviations: BAI, bioavailable iron; CFA, coal fly ash; CWP, coal workers' pneumoconiosis; DFO, deferoxamine mesylate; NSCWP, National Study of CWP; PBS, phosphate-buffered saline; RFU, relative fluorescence units; ROS, reactive oxygen species; TBA, thiobarbituric acid; TBARS, TBA reactive substances

INTRODUCTION

Coal mining causes health problems in coal workers. Although the dust standard of 2 mg/m^3 air had been established, medical evidence indicates that the risk to miners of developing coal workers' pneumoconiosis (CWP) is greater than had been previously predicted. Recently, the dust level had been lowered to 1 mg/m^3 air. However, this presents significant engineering challenges for the growing number of longwall mining techniques to attain the mandated level.[1] Moreover, chronic obstructive pulmonary diseases such as asthma and emphysema, indistinguishable from their non-occupational analogues, appear to be more relevant in contributing to the morbidity and mortality of coal workers.[2–4] It is now known that pneumoconiosis can become progressive after cessation of dust exposure, therefore, extensive at-risk populations consist of retired or ex-coal miners and those currently active in the

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workplace. The cost in disability benefits dictates that there is a need for surveillance and screening programs carefully monitoring early adverse effects and the health of those subjects. Knowing the active compounds, which are responsible for the toxicity in coal dust, will certainly help to develop such programs.

Despite comparable dust exposure levels, marked regional differences in the prevalence of CWP are apparent in the US.[5,6] In the first round of the National Study of CWP (NSCWP) conducted between 1969–1971, 31 coal seams from ten states were selected and over 9000 coal workers participated in the survey (90% participation rate).[5] After adjusting for the age of miners, the average tenure in mining, the job categories, and the estimated dust concentration and exposure, the prevalence of CWP was highest in Pennsylvania (PA) coal miners (26%), lower in West Virginia (WV) coal miners (10%), and lowest in Utah (UT) coal miners (4%).[5,7] The follow-up studies at the same mines (in 1972–1975, 1977–1981, and 1985–1988) have shown that the overall prevalence of CWP was decreased in coal miners, but the regional difference persisted with greater risk in Eastern coal miners (PA and WV) than in Western coal miners (UT and Colorado).[8]

Coal rank has been suggested to play a role in the regional difference in CWP because there is a rough correlation between coal rank and the prevalence of pneumoconiosis.[4,8] However, coal rank is only an indicator of the carbonization of organic materials in the coals and is not an active component, chemically speaking, which can induce cell injury that leads to the development of lung disease. The goal of the present study was to identify factor(s) in the coal responsible for coal workers' lung diseases occurring through oxidative stress mechanisms. We tested two hypotheses, (1) bioavailable iron (BAI, including both Fe^{2+} and Fe^{3+} ions) in coal dusts is the active component in inducing oxidative stress, and thus, contributes to coal dust-induced lung disease; and (2) calcite ($CaCO_3$), present in some of the coals, such as those from the UT coal mine region, plays a protective role in coal-induced oxidative damage by preventing acid solubilization of iron compounds, thus making iron less bioavailable. Therefore, the differences in the BAI content in the coals may be responsible for the observed regional differences in the prevalence of CWP. We examined the ability of coal samples from three coalmine regions (UT, WV, and PA) to release iron in a cell-free system (10 mM phosphate solution, pH 4.5), which mimics the conditions of the phagolysosomes of cells. We further examined this hypothesis using human lung epithelial Type II A549 cells, which provide a much more complex and efficient clearance system to release iron than the cell-free system we have been using. Our results strongly suggest that indeed a prolonged oxidative stress induced by BAI released from the PA coals is responsible for cellular damage that leads to the observed high prevalence of lung disease in the PA coal miners.

MATERIAL AND METHODS

Chemical Reagents

Ferrous sulfate heptahydrate, ferric sulfate pentahydrate, 2,2′-dipyridyl, deferoxamine mesylate (DFO), ammonium chloride, Tris buffer, *n*-butanol, glacial acetic acid, 2-thiobarbituric acid, sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Company (St. Louis, MO) with the highest purity available. Iron and calcium determination kits were also obtained from Sigma.

Sources of Coal Samples

Coal samples from three coalmine regions of UT, WV, and PA with different prevalence of CWP were purchased from the Penn State Coal Sample Bank as previously described.[9] We realized that the 12 coal samples from WV in our previous study were from two coal seams (six coal samples/seam),[9] which were probably not representative of the characteristics of the coals from WV region. Eight more coal

TABLE I Information on eight coal samples from WV coal mine region*

PSOC No.	Seams†	County	M/Yr	Sample type‡
130	Pocahontas #3	McDowell	5/68	Chan-Sect
702	Pittsburgh	Braxton	6/77	Chan-Seam
816	Pittsburgh	Harrison	7/77	Chan-Seam
895	Cedar Grove	Mingo	6/77	Chan-Seam
896	Cedar Grove	Mingo	6/77	Chan-Seam
997	Pittsburgh	Marion	5/77	Chan-Seam
1508	Pocahontas #3	McDowell	11/85	Chan-Seam
1519	Pittsburgh	Marion	6/86	Chan-Seam

* Information on other coal samples, namely those from PA and UT coal mine regions and two from WV, was published previously.[9] PSOC No. is the sample designation number from the Penn State Coal Sample Bank. † Location of the sample (seam and county) and time of the sample collected (month/year). Please note that samples may be from the same coal seam but different counties or the same coal seam and county but different years when samples were collected. ‡ Chan-Seam indicates full-seam channel samples. In general, 25–450 kg coals were collected and homogenized in accordance with appropriate American Standards for Testing Materials (ASTM) methods. This is the most useful sample type for various studies. If Chan-Seam is not available, Chan-Sect, which represents vertical sections of the seam, was chosen for the study.

samples from that region were ordered from the Penn State Coal Sample Bank. Table I lists the information on the origins, years collected, and sample types of these eight coal samples. As a result, in the present study a total of ten samples were included from the WV coalmine region. That is, two were from the two coal seams of the previous study and the eight were newly purchased. Therefore, we have more representative coal samples from West Virginia coalmine region.

Spectrophotometric Determinations of Bioavailable Fe^{2+}, Fe^{3+}, and Ca^{2+} in Aqueous Coal Filtrates

Before measurements, coal samples (approximately 5 g each) were ground in air in an agate mortar for 5 min. To determine the bioavailable Fe^{2+}, Fe^{3+}, and Ca^{2+} contents in the coals, 2.7 g of ground coal were suspended in 30 ml of 10 mM phosphate solution (pH 4.5). After incubation at room temperature with occasional stirring, 1 ml of the aqueous coal suspensions was taken at 3 h, 1, 3, and 7 days incubation. The suspensions were then filtered through a 0.65-μm membrane (Cellulose Acetate Membrane, Millipore, Bedford, MA) to remove coal particles. Fe^{2+} in the aqueous coal filtrates was detected by the formation of 2,2′-dipyridyl-Fe^{2+} complexes, which were monitored spectrophotometrically at 520 nm (Beckman). Fe^{3+} and Ca^{2+} were measured at 430 and 600 nm, respectively, by forming DFO-Fe^{3+} complexes and Ca^{2+}-Arsenazo III complexes. We have calculated that the highest level of ferric ions in our aqueous coal suspensions is about 1.7 mM (see Table II, 10,401 ppm in 2.7 g coal per 30 ml), below ferric phosphate precipitation level. Moreover, we did not observe any precipitation after mixing the aqueous coal extract from water with 10 mM phosphate. The quantities of bio-available Fe^{2+}, Fe^{3+}, and Ca^{2+} were determined by comparison with standard absorbance curves obtained using commercial $FeSO_4{\cdot}7H_2O$, $Fe_2(SO_4)_3{\cdot}5H_2O$, calcium phosphorus standard, and were presented as ppm of coal.

Measurements of Bioavailable Cr, Ni, Cu, and As in Coals by Atomic Absorption

Bioavailable Cr, Ni, Cu, and As contents that were released at the same time after coal acidification were determined by flame atomic absorption spectrophotometer via a standard curve of each element. The following parameters were used for AA analysis: Cu (wavelength 324.8 nm, bandpass 0.5 nm), Ni (232.0, 0.2 nm), As (193.7, 0.5 nm), and Cr (357.9, 0.5 nm).

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

Treatments of cells with particles require that the particle sizes are less than 10 μm in diameters, so that particles can be incorporated into the cells through phagocytosis by phagocytic cells or endocytosis by epithelial cells. Coal samples were ground and size-classified using the Mercer Impactor (Intox, Albuquerque, NM). Coal samples were ground in a ball mill with 5-mm diameter glass beads for 24 h. 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 over 80% of coal particles were less than 5 μm (data not shown). Coal dusts collected on the filters were stored in a desiccator at 4°C to inhibit microbial growth.

Cell Culture and Treatment With Two Coals Containing Different Levels of BAI

A human lung epithelial cell line, A549, with characteristics of alveolar epithelial type II cells, was obtained from American Type Culture Collection (ATCC CCL185). A549 cells were cultivated in a

TABLE II Average levels of bioavailable iron, calcium, and other metals in three coal mine regions with different prevalence of CWP*

Region	CWP†	Fe^{2+}	Fe^{3+}	Ca^{2+}	Ni	Cu	As	Cr
UT ($n = 10$)	4%	1.95 ± 1.93 (Range 0–19.3)	7.62 ± 2.21 (0–25.8)	717.5 ± 84.64 (286.4–1042)	0‡	0	0	0
WV ($n = 10$)	10%	1963.14 ± 886.90 (0–7535.9)	2695.7 ± 1192.7 (0–9897.8)	498.83 ± 131.5 (15.6–1249.9)	0.11 ± 0.062	0.17 ± 0.076	0.30 ± 0.20	0
PA ($n = 8$)	26%	7288.2 ± 2710.7 (543.8–20045.7)	4860.8 ± 1376.1 (672–10401.2)	662.9 ± 70.5 (531.1–1127.5)	0.4 ± 0.06	0.32 ± 0.074	0.13 ± 0.12	0

*Metal concentrations were parts per million (ppm) of coal (w/w). 2.7 g of coal samples were suspended in 30 ml phosphate solution (10 mM, pH 4.5) for 3 days, which mimics the phagolysosomal conditions of cells. Metals released under such conditions were considered as bioavailable. After filtration, levels of Fe^{2+}, Fe^{3+}, and Ca^{2+} in the coals were measured by spectrophotometer using 2,2′-dipyridyl, DFO, and Arsenazo III as their color-forming complexes, respectively. Levels of Ni, Cu, As, and Cr were determined by atomic absorption. Data are presented as mean ± standard error (SE) of the coal samples from each region. †Prevalence of CWP was from Refs. [5,7]. ‡Below detection limit.

75-cm^2 flask (Fisher Scientific, Fair Lawn, NJ) in 10 ml of Dulbecco Minimum Essential Medium (DMEM/F12) completed with 10% fetal bovine serum, 1% antibiotics, 1% L-glutamine and grown in an atmosphere of 95% air, 5% CO_2 at 37°C. At approximately 60% confluence, cells were treated with size-classified coal particles for various time points. Exposure to coal particles was expressed in $\mu g/cm^2$ because at these exposure conditions the particles were not completely soluble. The two dust samples from the PA and UT coal mine regions were added to the tissue culture media for cell treatments. As a negative control, each experiment included plates with cells to which nothing had been added. Following treatment, cell culture media were taken for Fe and Ca analysis. Cells were washed twice with phosphate-buffered saline (PBS) to remove coal particles, and cells were then dislodged with 0.5% Trypsin without EDTA. EDTA is a known iron chelator that may mobilize iron from coal particles and interfere with the iron measurements in cells. After centrifugation, cells were washed once more with PBS and cell viability was evaluated using Trypan blue exclusion assay. Cells were counted after each treatment using a hemocytometer and finally stored at −80°C for the following experiments.

Measurement of Fe and Ca in A549 Cells

Immediately prior to Fe and Ca measurements, control and coal-treated A549 cells were suspended at 2.5×10^6 cells/ml in a buffer containing 10 mM Tris buffer, pH 7.2 and 150 mM NH_4Cl, and lysed on ice and occasional vortexing. Phagocytized particulates of coal and cell debris were removed by centrifugation at 10,000*g* for 15 min. The supernatants were recovered and aliquoted for cellular Fe and Ca analyses, as well as protein determination. Protein concentrations were determined using Bradford reagents (Sigma). Cell precipitates (matrix) were used for lipid peroxidation assays.

The Fe levels in the cell supernatants were determined using commercially available kits (Sigma, St. Louis, MO). The principle of the test is that ferrozine, a sulfonated derivative of diphenyltriazine, forms a water-soluble magenta-colored complex with iron. At acidic pH and in the presence of suitable reducing agents, transferrin-bound iron dissociates to form Fe^{2+}. These Fe^{2+} ions, along with BAI, if present in cells, react with ferrozine to produce a magenta-colored complex with absorption maximum at 560 nm. The difference in color intensity at this wavelength, before and after the addition of ferrozine, is proportional to the concentration of iron in cells that is present in BAI and transferrin.

Cellular Ca levels were determined using a commercially available kit from Sigma. The principle of the test is that Ca in acidic environment forms a purple colored complex with Arsenazo III [2,2′-(1,8-dihydroxy-3,6-disulfonaphthylene-2,7bisazo) (-bis-bensenearsonic acid]. The intensity of the color, measured at 600 nm, is directly proportional to the Ca concentration in the sample. The results are expressed as nmoles Fe or μmoles Ca per 10^6 cells.

Measurements of Lipid Peroxidation

The levels of lipid peroxides in the coal-treated cells were measured by a thiobarbituric acid (TBA) reaction, as previously described.[10] Cell matrix samples were suspended in 0.1 ml PBS at 10^7 cells/ml and then mixed with 0.032 ml of 10% SDS and 0.6 ml of 0.4% TBA in 10% acetic acid, pH 5. The mixture was adjusted to 0.8 ml with distilled water, and then heated to 90°C for 1 h. After cooling, 0.8 ml of butanol was added and the mixtures were shaken vigorously. After centrifugation at 1500 rpm for 15 min, the fluorescence intensities of TBA-reactive substances (TBARS) in the butanol phase were measured at an excitation wavelength of 515 nm (bandwidth 4.5 nm), and an emission wavelength of 553 nm (bandwidth 9.0 nm) using a fluorescence-chemiluminescence microplate reader (Gemini, Molecular Device). The results for lipid peroxidation are expressed as relative fluorescence units (RFU) per 10^6 cells.

RESULTS

Bioavailable Iron, Calcium and Other Trace Metals in Aqueous Coal Filtrates

To test our hypotheses, we investigated the ability of a large number of coal samples from the various seams of three coalmine regions to release bioavailable metals at pH 4.5. Levels of BAI and calcium were slightly increased as function of incubation time from 3 h to 3 days and a drop at day 7 (data not shown). Table II shows data of day 3 that average levels of bioavailable Fe^{2+}, Fe^{3+}, Ni, and Cu are high in PA coals, lower in WV coals, and lowest in UT coals. Levels of bioavailable Cr was below detection limit (< 0.1 μg/ml) in all of the coal samples. Because of the heterogeneity of the coal samples, the standard deviations in each region are large as reflected by the wide range of each metal measured (Table II). Ca^{2+} is present in all coals, though the UT coals have the highest levels of Ca^{2+} released. The prevalence of CWP from the NSCWP in the 1970s were reported as 4, 10, and 26% in the coal miners of UT, WV, and PA, respectively.[5,7] Since most of the coal samples in the present study were collected during the same period when the epidemiological studies were carried out, we plotted the prevalence of CWP versus the amount of BAI in the coals (not shown). We found that

TABLE III Bioavailability of metals as a percentage released under acidic conditions mimicking phagolysosomes of cells*

Coal region	Fe % release†	Ca % release	Cu % release	Ni % release
UT ($n = 10$)	0.45 ± 0.29	13.95 ± 5.33	0‡	0
WV($n = 7$)¶	24.66 ± 14.57	20.33 ± 8.60	1.61 ± 0.84	2.58 ± 1.39
PA ($n = 8$)	42.28 ± 11.25	52.34 ± 7.66	1.27 ± 0.33	1.95 ± 0.43

* Total amounts of each metal were calculated from the data sheet provided by the Penn State Coal Sample Bank. Major elements as % (e.g. oxides as Fe_2O_3 and CaO) or trace elements as ppm (e.g. Ni, Cu, Cr, Zn, etc.) of high temperature ash and of total dry coal were available for most of coal samples. † % Release: amounts of metal released under acidic conditions (10 mM phosphate, pH 4.5) divided by the total amounts of that metal present in the coals × 100 (w/w). ‡ Level of the metal was below detection limits of atomic absorption spectrophotometer and 0% bioavailability was given. ¶ Mineral data from three coal samples of WV (PSOC# 895, 896, 997) were not available from the data sheet and, thus, not included for the analyses.

prevalence of CWP correlated very well with bioavailable Fe^{2+} content (correlation coefficient $r = 0.93$) after log transformation of the prevalence of CWP and Fe^{2+} levels in the coals. Correlation was also very good between prevalence of CWP and bioavailable Fe^{3+} ($r = 0.90$) or total BAI ($Fe^{2+} + Fe^{3+}$, $r = 0.92$). There were also positive correlations between prevalence of CWP and bioavailable Ni and Cu levels but not As levels in the coals. Since these bioavailable transition metals represent only a very small fraction of the inorganic compounds in the coals in comparison with the levels of BAI, we did not as yet perform any further studies on these transition metals, such as metallothionein induction in the cells.

Bioavailability of Iron in the Utah Coal Samples and Its Inhibition by Calcite

In this context, bioavailability is defined as the % (w/w) of the amount of metal released under conditions mimicking the cell lysosomes (10 mM phosphate solution, pH 4.5) divided by the total amount of that metal originally present in the coal. Table III shows that bioavailability as % release of Fe and Ca is the lowest in UT coal samples, moderate in WV coal samples, and highest in PA coal samples. UT coal samples had the highest level of total Ca in the coals (8526 ppm in dry coal), but the average % level of bioavailable Ca was low (only 13.95% of total Ca). This indicates that Ca in UT coals most likely originated from water insoluble calcite ($CaCO_3$), which can consume acid before iron compounds, such as siderite ($FeCO_3$). This conclusion was supported by the observation of a higher % release of Ca than % release of Fe in the UT coals (Table III).

To further test the hypothesis that the amount of acid available is rate-limiting in iron released from coal, one UT coal sample (PSOC# 459) was suspended in two acidic media, one at pH 4.5 (PBS), and the other in 50 mM HCl. Figure 1 shows an increase in pH and Ca concentration as a function of incubation time of the coal suspended in the PBS medium. Interestingly, levels of Ca^{2+} were gradually increased, and pH was immediately increased and then stabilized. There was no detectable iron in the aqueous coal suspension (data not shown). When the UT coal was suspended in 50 mM HCl, we found that this coal released 123 ppm Fe^{2+}. We then mixed UT coals with 50 mM HCl (2.7 g coal per 30 ml, pH 1.3), and an average level of 484 ± 103 ppm ($n = 10$) of Fe^{2+} was released in comparison to 1.95 ± 1.93 ppm released under standard acidic conditions (pH 4.5). These results strongly indicate that the UT coal samples contain iron compounds, which cannot be

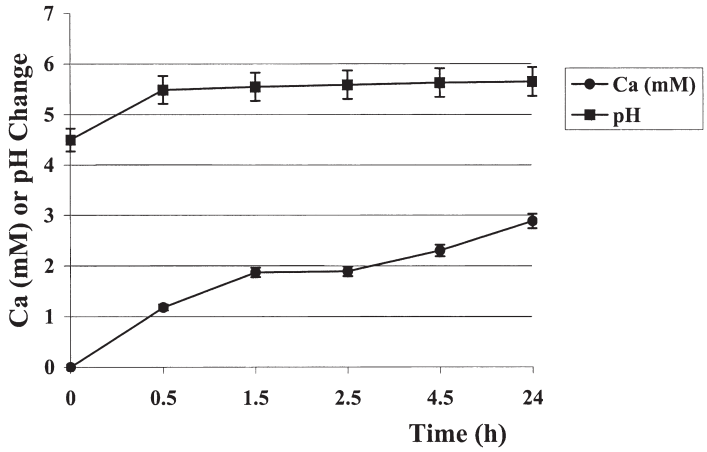

FIGURE 1 Increase of pH and Ca release as a function of incubation time of the UT coal (PSOC# 459) suspended in 10 mM PBS (pH 4.5). There was no detectable iron under this condition. Data were obtained from three independent experiments and are expressed as mean ± SE. The changes in pH and Ca were significantly different from time 0 by Student's t test ($p < 0.05$).

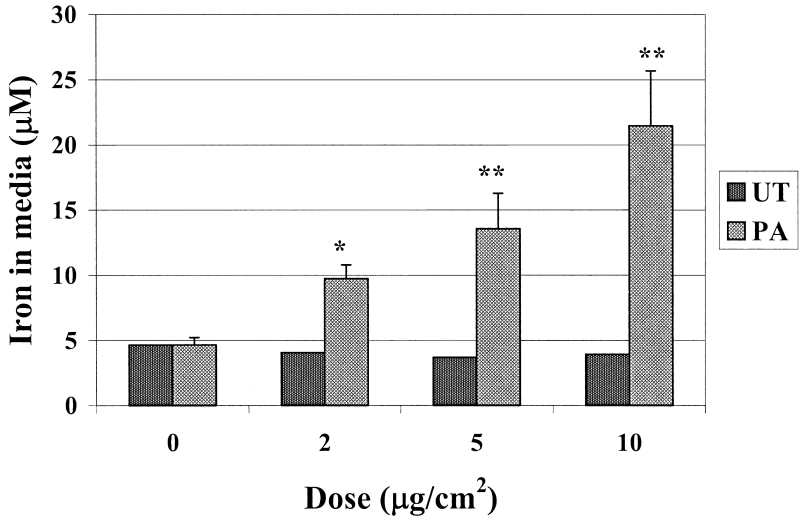

FIGURE 2 Release of iron from the PA coal into the tissue culture media. A549 cells plated in complete DMEM/F12 were treated with the PA (PSOC# 1198) or UT (PSOC# 459) coal particles at various doses for 24 h. After treatment, iron concentration in culture media was determined using ferrozine assay. The results were obtained from three independent experiments for the PA coal and two experiments for the UT coal (inter-assay correlation coefficient $r = 0.98$ between the two experiments). Data are expressed as mean ± SE. *: Significantly different from controls ($p < 0.05$) by Student's t test. **: Significantly different from controls ($p < 0.01$) by Student's t test.

released under physiologically relevant conditions because of the presence of calcite. Therefore, these data strengthen our hypothesis that calcite in the UT coals may play a protective role in coal-induced oxidative damage by inhibiting the acid solubilization of iron compounds in these coal samples. The average level of total Ca in PA coals was the lowest (1440 ppm, calculated from the data sheet), but it yielded a level of bioavailable Ca comparable to that in UT coals (Table II). In terms of bioavailability, 52.3% of Ca in PA coals was released. Therefore, the half of the Ca in PA coals may be in water-soluble forms, such as $CaSO_4$, which has no ability to counteract the leaching effect of acid on iron compounds in the PA coals.

Cellular Levels of Iron and Calcium in A549 Cells Treated With a Coal Dust from PA or UT Regions

A549 cells were treated with two coal samples, one from UT (PSOC# 459, bioavailable Fe^{2+} and Fe^{3+}: undetectable and 4.6 ppm), and the other from PA (PSOC# 1159, bioavailable Fe^{2+} and Fe^{3+}: 15010.4 and 8587.9 ppm, respectively). These coal samples are size-classified as described in the "Material and methods" section. After 24 h treatment, complete

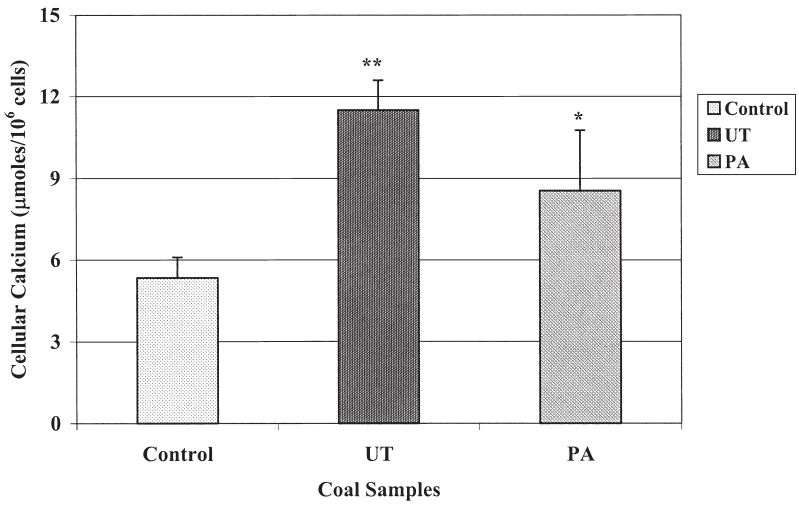

FIGURE 3 Cellular Ca levels in control, PA and UT coal-treated A549 cells. Cells were treated with the two coal samples at 2 μg/cm^2 for 24 h. The results were obtained from 4 independent experiments with the PA and UT coals and 7 experiments with the control samples. *: Significantly different from controls ($p < 0.05$) by Student's t test. **: Significantly different from controls ($p < 0.01$) by Student's t test.

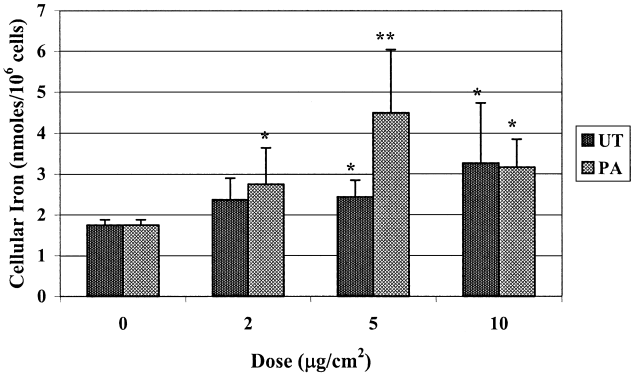

FIGURE 4 Cellular Fe levels in control, PA and UT coal-treated A549 cells as a function of dose. Cells were treated with the PA and UT coals for 24 h. The results were obtained from 3 independent experiments with the PA and UT coals at each dose and 9 experiments with the control samples. *: Significantly different from controls ($p < 0.05$) by Student's t test. **: Significantly different from controls ($p < 0.01$) by Student's t test.

tissue culture media were taken for iron measurements using ferrozine assay. Figure 2 shows a dose-dependent increase of iron in cell culture media treated with the PA coal but not in those treated with the UT coal. These results indicate that a part of the iron in the PA coal sample was released into the medium and became bioavailable for cellular uptake. The coal from UT had the same level of iron as the control medium completed with 10% serum. Of that amount of iron in the control medium ($4.66 \pm 0.57\,\mu M$), $1.7\,\mu M$ were from $FeSO_4{\cdot}7H_2O$ ($1.5\,\mu M$) and $Fe(NO_3)_3{\cdot}9H_2O$ ($0.2\,\mu M$) as essential components in the medium and the remaining iron was released from transferrin present in the 10% serum. Since the DMEM/F12 medium also contained 1.05 mM Ca^{2+}, no significant differences in levels of Ca^{2+} were observed in media treated with the UT and PA coals in comparison with control samples (data not shown). However, significant increases in cellular Ca levels were observed in A549 cells treated

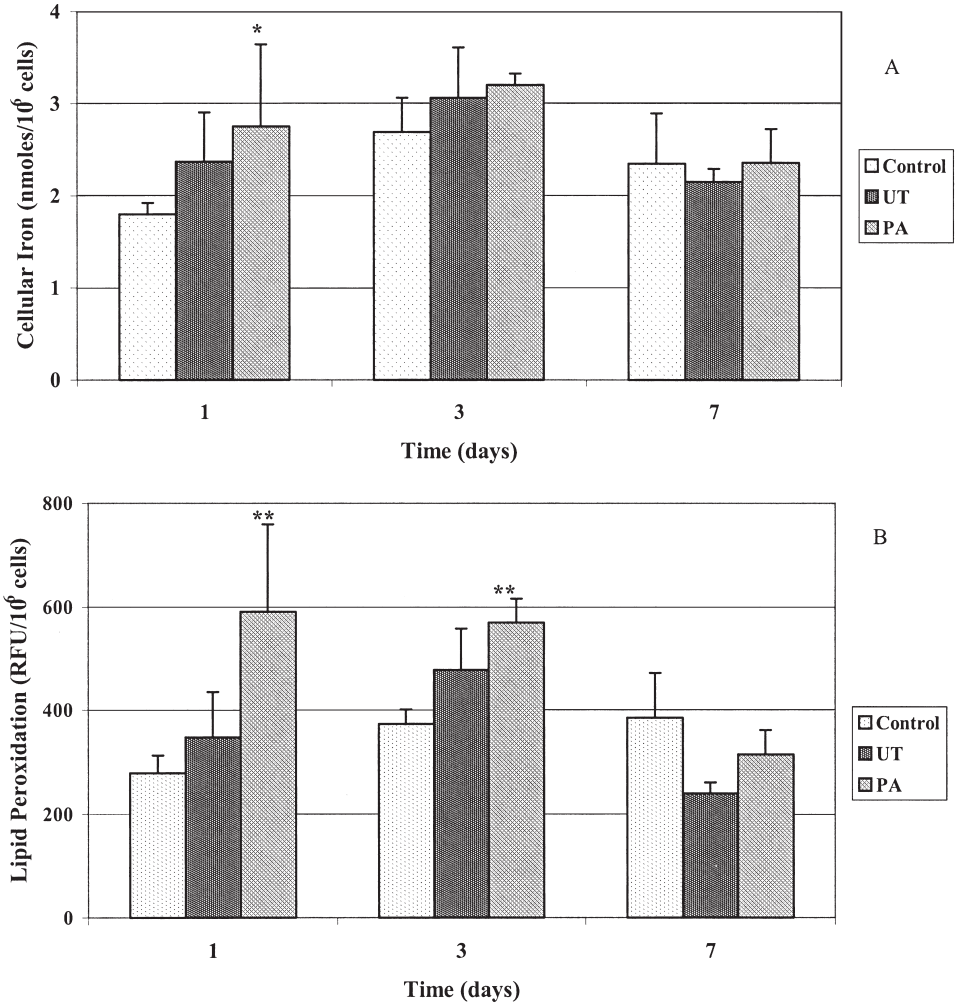

FIGURE 5 Cellular Fe (A) and lipid peroxidation (B) levels in control, PA and UT coal-treated A549 cells as a function of the exposure time. Cells were treated with the PA or UT coals at a dose of $2\,\mu g/cm^2$. After cell lysis, cell supernatants were used for Fe measurements (A) and cell precipitates (matrix) were used for lipid peroxidation assay (B). The results were obtained from 3 independent experiments with the PA and UT coals at each time point and 10, 4, and 3 experiments with the control samples at 1, 3, and 7 days, respectively. *: Significantly different from controls ($p < 0.05$) by Student's t test. **: Significantly different from controls ($p < 0.01$) by Student's t test.

with PA and UT coals at a dose of 2 $\mu g/cm^2$ (Fig. 3). The UT coal induced a 115% increase while the PA coal exhibited a 60% increase over the control. This was probably due to the endocytosis of coal particles by A549 cells and subsequent release of Ca from the coal particles into the cells.

Figure 4 shows significant increases in intracellular iron levels in A549 cells treated with PA and UT coals. The increase in cellular iron was more pronounced in PA coal-treated cells at a dose of 5 $\mu g/cm^2$ than 10 $\mu g/cm^2$. At 10 $\mu g/cm^2$ for 24 h, cells treated with PA or UT coals were 75% viable as measured by Trypan blue assay, while at 5 $\mu g/cm^2$, 80% of cells were viable (data not shown). It is interesting to note that the UT coal induced increases in cellular iron that were marginally significant at 5 and 10 $\mu g/cm^2$ ($p < 0.03$ and 0.04). Whether the iron in UT coal-treated cells was bioavailable needs further investigation, because measurements of iron in cells using the ferrozine assay include BAI and iron released from transferrin.

Cellular Iron and Lipid Peroxidation in A549 Cells Treated with PA and UT Coals as a Function of Time

Figure 5 shows cellular iron changes and induction of lipid peroxidation as a function of incubation time. At 2 $\mu g/cm^2$, the PA coal significantly increased iron levels in A549 cells after 1-day treatment (Fig. 5A). At 3 and 7 days, there were no significant changes in both PA and UT coal-treated cells over control cells. These results indicate that iron in the PA coal was readily bioavailable, and 1-day treatment provides sufficient time for PA coal to release iron into the cells.

Lipid peroxidation data (Fig. 5B) show that PA coal significantly increases levels of TBARS over those in control cells after 1-day treatment. The high level of lipid peroxidation was sustained up to 3 days, though the cellular iron was back to control levels within 3 days. At 7 days, lipid peroxidation returned to the normal as control levels. In contrast to the PA coal, the UT coal, which does not release BAI in the cell-free system, does not cause significant lipid peroxidation either. More interestingly, the UT coal had a more pronounced decrease in TBARS formation than the controls at 7-day treatment, bordering to statistical significance ($p < 0.09$).

DISCUSSION

Exposure to coal dusts results in pneumoconiosis as well as other lung diseases with initiation and progression of pulmonary fibrosis.[11] Epidemiological studies of the relationship between the prevalence of CWP and environmental measurements have consistently revealed that the predominant risk factor is respirable mixed coal dusts. Quartz was a minor contributor to CWP development in general.[12] Although improvements in working conditions and dust control measures in industrialized countries have led to a decrease in the incidence of severe forms of CWP, occupationally-related airway disease including asthma have emerged as having substantial public health importance.[13] In the past, pneumoconiosis has received the most attention because of its clear occupational association.

CWP is considered as one of the human lung pathologies related to oxidative stress, which is a disturbance in the oxidant/antioxidant steady state in favor of oxidants, thus leading to cellular damage. Previous studies on symptomatic coal miners have shown that alveolar macrophages, recovered from broncho-alveolar lavage, released excessive amounts of reactive oxygen species (ROS) and inflammatory cytokines.[14,15] A recent report showed that the antioxidant enzymes, as well as cytokines, growth factors, and fibronectin were significantly increased in the acellular broncho-alveolar lavage fluids of coal miners.[16]

The goal of the present study was to identify active component(s) in the coals that may induce secretions of ROS, inflammatory cytokines, and growth factors, leading to cell injury and initiating the disease process. The differences in the amounts of active component(s) in the coals may be responsible for the regional differences in the prevalence and severity of CWP. Our previous studies have indicated that acid soluble Fe^{2+} is the active component in the coals likely responsible for ROS formation and up-regulation of oxidant-responsive transcription factor activator protein-1 in the human tracheal epithelial cells.[9] Furthermore, it was shown that acid-soluble Fe^{2+} content in French coals correlates positively with prevalence of CWP in coal miners working in the coal mine regions where the coal samples were collected (correlation coefficient $r = 0.94$).[17] Since there were only five samples from four coal mine regions in that study, its significance was limited. Using 30 coal samples from the US,[9] the correlation between acid soluble Fe^{2+} content and prevalence of CWP was not as significant as we had expected. We have realized that the 10 coal samples from the WV coal mine region were collected from only two seams and were probably not representative of the whole region. In the present study, we expanded the coal seams from two to five in the WV regions while keeping the sample number at ten, similar to that of the PA and UT coal mine regions. We have measured not only bioavailable Fe^{2+}, but also bioavailable Fe^{3+}, Ca^{2+}, and other metals such as Cr, Ni, Cu, and Co. This covers a broader area of transition metals, which may play important roles in oxidant formation, and

thus contribute to coal dust-induced lung diseases. As a result of the samples from WV being more representative, the correlation between bioavailable Fe^{2+} and prevalence of CWP was greatly improved (correlation coefficient $r = 0.93$). More interestingly, we have shown for the first time that bioavailable Fe^{3+} may also be a strong contributor of CWP development. Because of the difficulties of producing large amounts of size-classified particles using the Impactor, measurements of bioavailable metals were carried out in coals ground in an agate mortar, all prepared in the same manner. Therefore, the actual levels of bioavailable metals in size-classified coal particles may be different from the ones ground in agate mortar, but it should not affect the regional differences in levels of bioavailable metals as we observed. These results are consistent with the epidemiological data and in very good agreement with our hypothesis that bioavailable metals, particularly iron (including Fe^{2+} and Fe^{3+}), may play an important role in CWP, and thus, contribute to the regional differences in the prevalence of the disease.

To further prove our hypothesis, we extended our study to an *in vitro* cell culture system. Human lung Type II A549 cell line was used for coal treatment in the present study because (1) Type II cells express proteases, which are likely to play a role in clearing fibrin deposits from the alveolar space in a number of forms of acute and chronic lung injury;[18,19] (2) although inflammatory events immediately following lung injury initiate the fibrotic process, the response of the pulmonary epithelium can be a major determinant of the pattern of tissue repair, and thus of the ultimate degree of permanent lung damage.[20]

Results of iron measurements in tissue culture media clearly indicate that iron release in the PA coal samples is dose-dependent, while iron levels in tissue culture media treated with UT coal were not significantly different from those present in control media (Fig. 2). These data suggest that iron in PA coal samples is bioavailable for cellular uptake. This was further supported by a dose-dependent increase in cellular iron levels (Fig. 4). It is interesting to note that the UT coal did not release iron in tissue culture media but enhanced cellular iron levels at 5 and $10\,\mu g/cm^2$. According to the lipid peroxidation data, the UT coal sample even at $10\,\mu g/cm^2$ did not induce significantly high levels of TBARS in A549 cells (477.0 ± 57.2 RFU/10^6 cells, $n = 4$) in comparison to the controls (518.9 ± 34.9 RFU/10^6 cells, $n = 3$). These results indicate that the increased iron in the UT coal-treated cells did not participate in oxidative processes, suggesting that this increase in cellular iron induced by the UT coal may be due to the upregulation of transferrin.

As we mentioned earlier about the possible protective effect of calcite in the UT coal by inhibiting acid solubilization of iron compounds, our *in vitro* assay showed a significant increase in Ca level but not in cellular iron level in A549 cells treated with the UT coal at $2\,\mu g/cm^2$ (Figs. 3 and 4). We have found that the UT coal used for cell treatment contains iron because it released 123 ppm of Fe^{2+} in 50 mM HCl. These results provide evidence that Ca from calcite is preferentially released in intact A549 cells as compared to iron compounds in the coal, such as siderite ($FeCO_3$). The average level of Ca in the UT coal-treated cells was also higher than that in the PA coal-treated cells, in parallel with the bioavailable Ca in our cell-free system (Table II). We also observed a decrease in TBARS in the UT coal-treated cells after 7-day exposure.

Although the decrease in comparison with the controls is not statistically significant ($p < 0.09$), we suspect that the UT coal may provide some protection from oxidative damage by releasing Ca^{2+} ions in cells.

It is also interesting to note that treatment of A549 cells with coal fly ash (CFA) obtained from UT coal resulted in a significant increase in mRNA levels of interleukin-8 (IL-8), a chemokine, as well as IL-8 protein.[21] For the same particle size (e.g. $<1\mu m$) and the same dose treatment ($40\,\mu g/cm^2$), CFA from UT coal had a more pronounced effect on IL-8 than the CFAs from Illinois and North Dakota coals. These effects were attributed to the properties of BAI present in the CFA of the UT coals. One of the differences between coal and CFA in the UT coal is the decomposition of calcite in CFA. As we suggested before, calcite in the coal can serve as a buffering component by consuming acid in the cells, and prevent iron compounds from becoming bioavailable, thus keeping the coal samples in a relatively neutral pH environment.[9] The buffering capacity of CFA was diminished due to the decomposition of calcite at high temperature, while iron in the CFA from UT coals may readily become bioavailable for ROS generation and subsequent production and release of inflammatory cytokines. These data suggest that a coal during mining does not have to cause health problems to coal workers if contains sufficient calcite. At the same time, it does indicate that the CFA generated by the same coal from power plants can be more hazardous to the general public, because changes in BAI levels may alter the toxicity profiles of the dusts.

In conclusion, results presented here strongly indicate that BAI in the coal is the active component in inducing oxidative stress in human lung epithelial cells, which may contribute to the development of pneumoconiosis. Calcite present in certain coals can prevent acid solubilization of iron compounds, and thus, may play a protective role in coal dust-induced lung disease. The measurements of BAI and other metals in the cell-free system correlated well with the

in vitro measurements in A549 cells. This study provides evidence that BAI in the coal may be used as a predictor of coal's toxicity applicable before mining.

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