Articles

Buffering Capacity of Coal and Its Acid-Soluble Fe^{2+} Content: Possible Role in Coal Workers' Pneumoconiosis

Xi Huang,*,† Jeanine Fournier,‡ Karen Koenig,† and Lung Chi Chen†

Department of Environmental Medicine, New York University Medical Center, 550 First Avenue, New York, New York 10016, and Laboratoire de Reactivite de Surface, Universite P et M Curie, 4 Place Jussieu, Paris, France

Received August 22, 1997

Downloaded via CENTERS DISEASE CONTROL & PRVNTN on March 4, 2019 at 15:13:56 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

Epidemiological studies have shown that the prevalence of coal workers' pneumoconiosis (CWP) differed remarkably between different coal mine regions despite comparable exposures to respirable dust. In the United States, CWP was found to be most common in Pennsylvania coal miners and least common in miners from Utah. The active component(s) responsible for the regional differences in CWP has not yet been identified. In the present study, we found that coals from Pennsylvania, compared with Utah coals, showed a much lower buffering capacity as determined by the amount of acid consumed in order to reach pH 4.5, which is the pH of the phagolysosomes of macrophages. Moreover, the coals from Pennsylvania released large amounts of Fe^{2+} in the acidified extract, whereas the coals from Utah released little Fe^{2+}. Using electron spin resonance (ESR), we found that the coals from Pennsylvania, but not from Utah, were effective in oxidizing formate by a radical pathway. Two coals, one from Utah with high buffering capacity and low acid-soluble Fe^{2+} and the other from Pennsylvania with low buffering capacity and high acid-soluble Fe^{2+}, were then selected for cell treatment. We found that human tracheal epithelial (HTE) cells treated with the coal from Pennsylvania (10 μg/cm^2) showed a 36% increase in oxidant formation over the control as detected by dichlorofluorescein assay, whereas the coal from Utah had no effect. An electrophoretic mobility shift assay was used to test the binding affinity of nuclear proteins extracted from the coal-treated HTE cells to an oxidative stress-responsive transcription factor activator protein-1 (AP-1) element. The coal from Pennsylvania with high acid-soluble Fe^{2+} (1 μg/cm^2) activated AP-1 to the same extent as 10 μM H_2O_2, while the coal from Utah without acid-soluble Fe^{2+} had no effect. These results support our hypothesis that the prevalence of CWP may be higher in coal workers exposed to coal with high acid-soluble Fe^{2+} and low buffering capacity than in workers exposed to coal with low acid-soluble Fe^{2+} and high buffering capacity.

Introduction

Epidemiological studies of mortality and morbidity in coal workers have identified three pathologies that are related to coal dust inhalation: pneumoconiosis including simple pneumoconiosis and progressive massive fibrosis (PMF),[1] chronic obstructive bronchopneumopathy such as emphysema and/or chronic bronchitis, and stomach cancer (*1*). Among the respiratory diseases, coal workers' pneumoconiosis (CWP) has received the most attention because of its clear occupational association. Bronchitis and emphysema resulting from coal mine dust exposure, clinically indistinguishable from their nonoccupational analogues, are more prevalent and are associated with significant morbidity among coal workers.

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 (*2*). Although CWP was originally thought to be a variant of silicosis, coal mine dust usually contains relatively small amounts of free crystalline silica (quartz). Quartz has been found to be a minor contributor to CWP development in general (*3*, *4*). Moreover, pneumoconiosis was also found in coal trimmers who shoveled coal that contained little or no silica and in graphite and carbon electrode workers who were exposed to carbonaceous materials free of silica (*5*, *6*). Iron, one of the most abundant pollutants in the industrial environment, was

* Correspondence and requests for reprints should be addressed to Xi Huang, Ph.D., Nelson Institute of Environmental Medicine, New York University Medical Center, PHL Rm 802, 550 First Ave, New York, NY 10016. Tel: (212) 263-6609. Fax: (212) 263-6649. E-mail: xihuang@charlotte.med.nyu.edu.

† New York University Medical Center.

‡ Universite P et M Curie.

[1] Abbreviations: AMs, alveolar macrophages; AP-1, activator protein-1; CWP, coal workers' pneumoconiosis; DCF, dichlorofluorescein; DCF-dAc, dichlorofluorescein diacetate; DCFH, dichlorofluorescein; DMPO, 5,5′-dimethyl-1-pyrroline *N*-oxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) tetraacetic acid; ESR, electron spin resonance; HTA, high-temperature ashes; HTE, human tracheal epithelial; PMF, progressive massive fibrosis; PMSF, phenylmethanesulfonyl fluoride; ROS, reactive oxygen species.

S0893-228x(97)00151-3 CCC: $15.00 © 1998 American Chemical Society
Published on Web 06/02/1998

not examined in the above studies.

It has been shown in the United States, Great Britain, France, and Germany that the prevalence and severity of CWP differed remarkably between different coal mines despite comparable exposures to respirable dust (*7*−*10*). For example, the first round of the U.S. National Study of CWP, which was completed in 1971, has examined a total of 9076 miners from 29 bituminous and 2 anthracite mines (*11*). The average exposure concentration in U.S. mines during that period was 3 mg/m^3. It was found that 41% of the eastern Pennsylvania anthracite miners had simple pneumoconiosis and a further 14% had PMF, but the comparable figures for bituminous miners in Colorado and Utah were 4% and 0.4% (*11*, *12*). Followup studies at the same mines (in 1972−1975, 1977−1981, and 1985−1988) have shown that this regional difference persisted with greater risk of CWP in eastern coal miners (Pennsylvania and West Virginia) than in western coal miners (Utah and Colorado) (*13*). In France, coal miners of Provence never had CWP (0%), while the prevalence of CWP in coal miners of Nord Pas de Calais was 24% (*9*). In Great Britain, a detailed study of eight mines has shown that one mine with an exposure concentration of 9.1 mg/m^3 (pre-1970 dust conditions) reported 2.4% of CWP and another mine with only 3.6 mg/m^3 reported 16.6% of CWP (*14*). Coal rank was found to play a role: risk increases with the coal rank (*1*). Coal rank is defined as the extent to which the organic materials have matured during geological time. The four major coal types ranked in order of increasing heat value are: lignite < subbituminous < bituminous < anthracite. Coal rank can be roughly estimated by the carbon content in the coal, molar ratio of carbon/hydrogen (C/H), heat value, volatile materials, or moisture (*15*). A correlation between coal rank and cell cytotoxicity has not yet been established in biological studies (*16*). It has been suggested that the physicochemical characteristics of the coal mined may be responsible for regional differences in the prevalence of CWP.

We hypothesize that acid-soluble Fe^{2+} may be the active compound in inducing CWP. There are strong indications that the ability of alveolar macrophages (AMs) to dissolve inorganic particles is due to the low pH in the phagolysosomes (*17*−*19*). One of the physicochemical parameters that can influence the dissolution of a particle is its buffering capacity, which can be defined as its ability to maintain its pH when in aqueous suspension. Acid-soluble Fe^{2+} in low-buffering-capacity coal would dissolve in the phagolysosomes of macrophages following phagocytosis and then induce oxidative stress leading to lung injury and CWP development. In contrast, high-buffering-capacity coal may prevent acid solubilization of Fe^{2+} and thus render the coal less hazardous. The goal of the present study is to determine the roles of acid-soluble Fe^{2+} content and buffering capacity of coal in the development of CWP. Thirty coal samples from three regions with different prevalences of CWP were studied. We found that the samples from the Pennsylvania coal mines with high prevalence of CWP have high acid-soluble Fe^{2+} content and low buffering capacity. In contrast, the samples from the Utah coal mine have a low content of acid-soluble Fe^{2+}, but very high buffering capacity. The coals from southwestern West Virginia had low buffering capacity but a moderate level of acid-soluble Fe^{2+}. These results are in agreement with our observation that the prevalence of CWP in France correlates positively with the acid-soluble Fe^{2+} content of the French coal samples (personel communication). This study further strengthens our hypothesis that the risk of CWP is higher in coal workers exposed to coal with high acid-soluble Fe^{2+} and low buffering capacity.

Materials and Methods

Chemicals. 2,2′-Bipyridine, $FeSO_4 \cdot 7H_2O$, 5,5′-dimethyl-1-pyrroline *N*-oxide (DMPO), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), ethylene glycol bis(*β*-aminoethyl ether) tetraacetic acid (EGTA), phenylmethanesulfonyl fluoride (PMSF), and sodium dihydrogen phosphate were purchased from Sigma. Dichlorofluorescein diacetate (DCF-dAc) was from Kodak, and sodium formate was obtained from Merck.

Measurements of pH, Buffering Capacity, and Acid-Soluble Fe^{2+} Content in Coals. Thirty bituminous coal samples from three regions with different prevalences of CWP were purchased from the Penn State Coal Sample Bank. In general, 50−1000 lbs of coals were freshly collected by the Penn State Coal Sample Bank and then ground, homogenized, and sealed under argon in foil/polyethylene laminate bags. This was to ensure that the analytical procedures which aimed at generating the basic compositional characterization required by coal investigators were performed in accordance with appropriate American Standards for Testing and Materials (ASTM) methods. This was also to ensure that the collected coal samples were not exposed to oxygen, which leads to chemical changes in the samples.

Upon receipt, the coals sealed under argon in foil/polyethylene bags were divided into three Qorpak bottles (Fisher Scientific) under dry nitrogen. One part of each coal sample was immediately ground in air in an agate mortar for 5 min. pH, buffering capacity, and acid-soluble Fe^{2+} of coals were measured in a blinded manner. Buffering capacity of coals can be defined in our context as the necessary volume (μL) of 1 M H_2SO_4 to reduce the pH of aqueous coal suspension (2.7 g of coal in 30 mL of distilled water) to pH 4.5. The initial pHs of aqueous coal suspensions were recorded with a Fisher Accumet pH meter before acidification. To determine the acid-soluble Fe^{2+} content of coals, 2.7 g of coal was prewashed in distilled water and then suspended in 30 mL of 10 mM phosphate solution (pH 4.5) containing 5 mM 2,2′-bipyridine, which is a chelator of Fe^{2+}. After 3 h of incubation at room temperature, the aqueous coal suspensions were then filtered through a 0.65-μm membrane (Cellulose Acetate, Millipore) to remove coal particles. Fe^{2+} in the aqueous coal filtrates was detected by the formation of 2,2′-bipyridine−Fe^{2+} complexes which can be analyzed by spectrophotometer (520 nm, Beckman). The quantity of Fe^{2+} was determined by comparison with a standard OD curve obtained using commercial $FeSO_4 \cdot 7H_2O$ (Sigma) and is presented as ppm of coal.

Detection of Reactive Oxygen Species (ROS) in Aqueous Coal Suspensions. The oxidative activities of 10 coal samples (5 from the Pennsylvania coal mines and the other 5 from the Utah coal mines) were examined by electron spin resonance (ESR) techniques. An experimental protocol was designed to test for the presence and reactivity of ROS (symbolized as A*) by measuring its ability to react with the formate anion. The principles of the experimental reactions are as follows:

$$A^* + HCO_2^- \rightarrow AH + CO_2^{-\bullet} \quad (1)$$

$$DMPO + CO_2^{-\bullet} \rightarrow DMPO{-}CO_2^{-\bullet} \quad (2)$$

The carboxylate radical anion ($CO_2^{-\bullet}$), whose lifetime is on the order of 10^{-9} s, can be trapped if formed in solution by a spin-trapping agent, DMPO. This creates a radical adduct (DMPO−$CO_2^{-\bullet}$), whose lifetime is at least 1 h. The quantity of formed ROS is evaluated by the intensity of the ESR signal of

DMPO−$CO_2^{-\bullet}$ (*20*). Acidified coal filtrate (1 mL) (0.9 g of coal in 10 mL of 10 mM phosphate solution, pH 4.5, for 1-h incubation) was added to a reaction (total volume of 2 mL) containing 1 M sodium formate and 50 mM DMPO in 250 mM sodium dihydrogen phosphate buffer (pH 7.4). Aliquots were withdrawn 5 min after the addition of all reagents. Blanks contained everything except coal extracts. The detection of the radical adduct DMPO−$CO_2^{-\bullet}$ was performed by ESR (Varian CSE instrument).

Formation of Fluorescent Dichlorofluorescein in HTE Cells Induced by Coal Dusts. A fluorescence assay for the detection of oxidant formation in living cells was used as previously reported (*21*). The principle of the test is based upon nonpolar DCF-dAc diffusing into the cells through the cell membrane. The DCF-dAc is then hydrolyzed to polar dichlorofluorescein (DCFH) by esterases and thereby trapped within the cells. In the presence of peroxidase and H_2O_2 (or other oxidants), DCFH can be oxidized to a fluorescent dichlorofluorescein (DCF). Human tracheal epithelial (HTE) cells were seeded at (3−4) × 10^5 cells in 60-mm dish in 5 mL of complete α-minimum essential medium (α-MEM; JRH Biosciences, Lenexa, KS). After 2 days, cells were treated with two coal samples at a concentration of 10 μg/cm^2. These two coals which were representative of two coal mine regions had different acid-soluble Fe^{2+} contents and buffering capacities. One was from a Utah coal mine (PSOC no. 1502, buffering capacity 505 μL, acid-soluble Fe^{2+}: undetectable), the other from a Pennsylvania coal mine (PSOC no. 1516, buffering capacity 0 μL, acid-soluble Fe^{2+}: 1202 ppm). These two coals were freshly ground under air and sieved to 5 μm. After 5 h of treatment with coal, cells were treated further with 50 μM DCF-dAc for an additional hour. Following treatment, cells were washed twice in ice-cold phosphate-buffered saline (PBS) and scraped with a rubber policeman. Cells were finally suspended in cold PBS to give a concentration of 1 × 10^6 cells/mL for measurements of fluorescence. The fluorescence was monitored on a Spex Fluorolog instrument (Edison, NJ), with excitation wavelength at 502 nm (bandwidth 1.8 nm) and emission wavelength at 522 nm (bandwidth 1.8 nm). Fluorescence intensity is presented as actual meter reading divided by 100 000.

Measurements of Activator Protein-1 (AP-1) in HTE Cells by Gel Mobility Shift Assays. Nuclear extracts were prepared as previously reported (*22*). In brief, control and coal-treated HTE cells were scraped with a rubber policeman, resuspended in hypotonic buffer A (10 mM Hepes, pH 8.0, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 10 μg/mL leupeptin, antipain, aprotinin, benzamidin, and pepstatin) for 15 min on ice, and then vortexed for 10 s with 0.5% NP-40. Nuclei were separated from cytosol by centrifugation at 12000*g* for 1 min, were resuspended in buffer C (20 mM Hepes, pH 8.0, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 10 μg/mL leupeptin, antipain, aprotinin, benzamidin, and pepstatin), and shaken for 15 min on ice. Nuclear extracts were obtained by centrifugation at 12000*g* for 5 min, and protein concentration was measured by Lowry's test following manufacturer's instructions (Bio-Rad). Double-stranded consensus oligonucleotide sequences for AP-1 were purchased from Promega (Madison, WI) and were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The labeled oligonucleotides were incubated with 6 μg of nuclear extracts in 25 μL of total reaction volume containing buffer C and 2.5 μg of poly(dI-dC) for 1 h on ice. The mixture was subjected to electrophoresis in a 4% nondenaturing polyacrylamide gel. The gel was then exposed to X-OMAT AR2 film (Eastman Kodak Co., Rochester, NY).

Results

Selection of Coal Samples. The coal samples for the present study were selected based on the published epidemiological data which reported different prevalences of CWP for specific coal seams in specified counties and states (*12*, *23*). We checked if the coal samples from the same seam, county, and state were available in the Penn State Coal Sample Bank. The Penn State Coal Sample Bank currently contains over 1100 well-documented coal samples. All of the Penn State coals are intended to provide characterized materials for scientific research. We chose the full-seam channel samples (Chan-Seam), channel samples of seams being mined (Chan-Work), or seam subsections (Chan-Sect), because they are the most representative of the coal seams among the coal samples which were collected. The time periods during which the coals were sampled ranged from the 1970s to the 1980s. We did not use respirable coal mine dusts collected by a coal sampler, because these respirable dusts were usually exposed to air for months. Our understanding is that coal dusts exposed to air for months can lose their oxidizing activity, and hence biological activity. This resulted in the selection of 30 coal samples from 8 seams of 3 states with reported prevalences of CWP of 4% for Utah (*23*), 10% for West Virginia, and 26% for Pennsylvania (*12*) (Table 1). All selected coals in the present study were bituminous.

Buffering Capacities, pHs, and Acid-Soluble Fe^{2+} in U.S. Coals. After obtaining the coal samples from the Penn State Coal Sample Bank, we examined whether the coal samples were homogeneous and representative and whether our measurements were reproducible. We found that different measurements of the same sample were very reproducible when a minimum of 0.5 g of coal was used (data not shown).

Table 1 shows that the coals from central Pennsylvania (PA), on average, released 2500 ppm of Fe^{2+} at pH 4.5 (SD 2300, $n = 8$). These coals were acidic and thus had little buffering capacity. Epidemiological studies have shown that CWP is frequent (26%) in the coal workers of central Pennsylvania. In contrast, coals from Utah (UT) were neutral or basic in aqueous medium. On average, these coals needed 469.2 μL of 1 M H_2SO_4 (SD 412.8, $n = 10$) to reduce the pH of aqueous coal suspensions to 4.5 and had little acid-soluble Fe^{2+} (0.3 ppm). A CWP prevalence of 4% was reported in the coal workers of Utah. The coals from southwestern West Virginia (WV), with an intermediate prevalence of CWP (10%), had low buffering capacity but a moderate level of acid-soluble Fe^{2+} (11 ppm). These results demonstrate that the prevalence of CWP correlates positively with the acid-soluble Fe^{2+} content in the U.S. coal samples. No such correlation was observed between the prevalence of CWP and the content of silica in the coals (Table 1).

From Table 1, it can be seen that measurements of the three sample types (Chan-Seam, Chan-Work, Chan-Sect) were similar. There are variations in the buffering capacities and acid-soluble Fe^{2+} contents of different coals from the same seam (e.g., compare the average buffering capacities of four coal samples from the Hiawatha seam in Utah). There are also variations among coals from different seams of the same coal mine region (e.g., compare the seams of Hiawatha, Upper Hiawatha, Lower Sunnyside, and Blind Canyon in Utah). However, the intraregion variation is small compared with the difference between regions.

Persistence of Acid-Soluble Fe^{2+} and Buffering Capacity over Time. Table 1 reveals no systematic change in buffering capacity and acid-soluble Fe^{2+} in the coals collected over a period of more than 10 years. The buffering capacities of four coal samples from the Hia-

Table 1. pHs, Buffering Capacities, and Acid-Soluble Fe^{2+} Contents of 30 Coal Samples from 3 Coal Mine Regions with Different Prevalence of CWP[a]

PSOC no.	seams	county	m/yr	sample type	pH	buffering capacity (μL)	Fe^{2+} (ppm) at pH 4.5	SiO_2 (%)
UT (4% CWP)								
313	Hiawatha	Carbon	7/74	Chan-Seam	7.98	150	0[b]	10.91
498	Hiawatha	Emery	8/76	Chan-Sect	6.16	240	0	3.86
500	Hiawatha	Emery	8/76	Chan-Sect	8.70	65	0	5.41
1502	Hiawatha	Sevier	9/85	Chan-Work	8.76	505	0	4.24
433	Upper Hiawatha	Servier	6/76	Chan-Sect	7.55	342	0	3.87
432	Upper Hiawatha	Servier	6/76	Chan-Sect	7.50	1455	3	3.82
431	Upper Hiawatha	Servier	6/76	Chan-Work	7.39	985	30	3.69
429	Upper Hiawatha	Servier	6/76	Chan-Seam	7.12	505	0	9.26
459	Lower Sunnyside	Emery	6/76	Chan-Seam	7.37	160	0	3.63
1112	Blind Canyon	Emery	8/78	Chan-Seam	6.38	285	0.4	3.56
mean ± SD						469.2 ± 412.78	0.33 ± 0.89	5.23 ± 2.51
WV (10% CWP)								
827	Sewell	Nicholas	7/77	Chan-Sect	6.65	5	0	1.42
826	Sewell	Nicholas	7/77	Chan-Sect	4.48	0	0	0.98
825	Sewell	Nicholas	7/77	Chan-Sect	4.18	0	51	2.89
824	Sewell	Nicholas	7/77	Chan-Seam	5.32	2	12	2.31
823	Sewell	Nicholas	7/77	Chan-Seam	6.58	5	0.9	1.75
822	Sewell	Nicholas	7/77	Chan-Seam	5.69	2	3	2.14
731	Sewell B	Randolph	6/77	Chan-Sect	6.05	2	4	2.60
730	Sewell B	Randolph	6/77	Chan-Sect	4.69	2	20	1.84
729	Sewell B	Randolph	6/77	Chan-Sect	6.97	2	4	0.84
728	Sewell B	Randolph	6/77	Chan-Seam	6.21	5	0.4	1.47
727	Sewell B	Randolph	6/77	Chan-Seam	5.48	3	60	1.73
726	Sewell B	Randolph	6/77	Chan-Seam	4.23	0	25	2.41
mean ± SD						2.33 ± 1.87	11 ± 15	1.87 ± 0.60
PA (26% CWP)								
260	Middle Kittanning	Clearfield	3/73	Chan-Seam	4.54	2	509	3.47
324	Middle Kittanning	Clearfield	9/74	Chan-Seam	3.33	0	2546	17.58
325	Middle Kittanning	Armstrong	10/74	Chan-Seam	1.95	0	5245	4.4
337	Lower Kittanning	Jefferson	4/75	Chan-Seam	3.15	0	1677	4.54
1197	Lower Kittanning	Somerset	11/79	Chan-Seam	3.56	0	1477	4.73
1198	Lower Kittanning	Indiana	11/79	Chan-Seam	2.67	0	6877	3.50
1313	Lower Kittanning	Clearfield	6/80	Chan-Seam	2.47	0	806	2.59
1516	Lower Kittanning	Somerset	5/86	Chan-Seam	3.30	0	1202	4.28
mean ± SD						0.25 ± 0.71	2500 ± 2300	5.63 ± 4.56

[a] PSOC no., sample designation number from the Penn State Coal Sample Bank; m/yr, month/year the coal was sampled; Chan-Seam, full-seam channel samples; Chan-Work, channel samples limited to the vertical extent of the seam being mined; Chan-Sect, channel samples of seam subsection; pH, pH of suspensions of coal samples in distilled water (2.7 g in 30 mL of H_2O); buffering capacity, microliters of 1 M H_2SO_4 needed to reduce the pH of the above aqueous coal suspension to pH 4.5; acid-soluble Fe^{2+}, ppm of coal released in 10 mM phosphate solution, pH 4.5. Data on SiO_2 content are provided by the Penn State Coal Sample Bank and are presented as percent of whole dry coal. [b] Not detectable.

watha seam in Utah which were collected from 1974 to 1985 were 150, 240, 65, and 505 μL of 1 M H_2SO_4, respectively, and no acid-soluble Fe^{2+} was detected in any of these samples. The acid-soluble Fe^{2+} contents in the samples from the Lower Kittaning seam in Pennsylvania which were collected from 1975 to 1986 likewise showed no systematic change over time, and no buffering capacity was observed in these coal samples.

Correlation between Buffering Capacities and CaO Contents in High-Temperature Ashes of Coals. We have previously observed that coal from the Gardanne mine of Provence, France, had a high buffering capacity. This coal contained a large amount of calcite ($CaCO_3$) which can neutralize acid (*24*). From the information sheet provided by the Penn State Coal Sample Bank, we obtained the CaO content in the high-temperature ashes (HTA) of each coal and then plotted them versus the buffering capacity that we measured. Figure 1 shows a high correlation between buffering capacity and CaO content (correlation coefficient r = 0.84). Thus, calcite, a primary source of CaO, might play an antagonistic role in coal dust-induced cell toxicity.

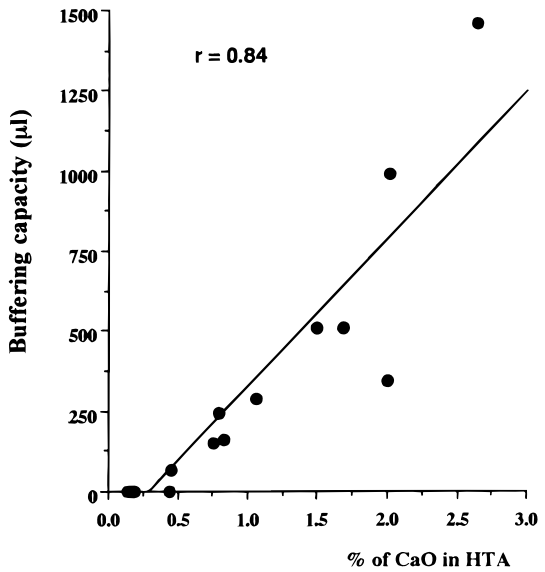

Figure 1. Correlation between the buffering capacities of coals and CaO contents in high-temperature ashes (HTA) of coals.

Oxidative Activities of the Samples from Pennsylvania and Utah Coal Mines. Table 2 shows that the five samples from central Pennsylvania coal mines with high prevalence of CWP were very active in oxidizing formate. In the presence of desferoxamine or in the

Table 2. Oxidative Activities of Coals as Detected by the ESR Signal DMPO−$CO_2^{-\bullet}$

region	seam	DMPO−$CO_2^{-\bullet}$ (a.u.),[a] 5 min
UT (4% CWP)		
1502	Hiawatha	<20[b]
500	Hiawatha	<20
313	Hiawatha	<20
431	Upper Hiawatha	<20
1112	Blind Canyon	60
PA (26% CWP)		
260	Middle Kittanning	815
324	Middle Kittanning	3500
1197	Lower Kittanning	980
1313	Lower Kittanning	1940
1516	Lower Kittanning	1365
blank		<20

[a] Varian CSE instrument, field set 3340 G, scan range 100 G, microwave power level 10 mW, time constant 1 s. Constants for DMPO−$CO_2^{-\bullet}$: $g = 2.0055$, $a_N = 15.6$ G, $a_H = 19.0$ G. 1000 au corresponds to 4.14×10^{18} radicals/L. [b] Average of two experiments.

absence of O_2, no ESR signal was observed in the aqueous filtrates of Pennsylvania coals, indicating that iron and oxygen are essential for the formation of oxidizing species. In contrast, four of the five coal samples from Utah mines did not show any detectable oxidizing activity under the same experimental conditions. The other sample (PSOC no. 1112) had a very weak activity. Our results demonstrate that coal dusts containing Fe^{2+} are able to form oxidants in aqueous media.

Effect of Coal Dusts on the Formation of DCF in Human Tracheal Epithelial (HTE) Cells. Although we show above that the coals from Pennsylvania mines were able to oxidize formate in a cell-free aqueous medium, it is not known whether these coals can increase oxidant formation in living cells. Two coals with different buffering capacity and acid-soluble Fe^{2+} were used for cell treatment. One was from Pennsylvania (PSOC no. 1516, buffering capacity 0 μL, acid-soluble Fe^{2+}: 1202 ppm), and the other was from Utah (PSOC no. 1502, buffering capacity 505 μL, acid-soluble Fe^{2+}: undetectable). HTE cells were treated with these two coals at a concentration of 10 μg/cm^2 for 5 h, followed by an additional 1-h treatment with DCF-dAc (50 μM) (*21*). Figure 2 shows that, for the same exposure concentration, a 36% increase in fluorescence over the control was observed in cells treated with the coal of Pennsylvania. The coal from the Utah mine had no effect on the fluorescence formation. These results indicate that the coal from Pennsylvania increased the oxidant formation in intact HTE cells.

Activation of Oxidative Stress-Responsive Transcription Factor AP-1 by Coal Dusts. NF-κB and AP-1 are inducible transcription factors that can be activated when cells are exposed to ROS. They control the expression of multiple antioxidative enzymes in response to H_2O_2 and $O_2^{-\bullet}$ (*25*). HTE cells were treated with the same two coals at a concentration of 1 μg/cm^2 for 5 h. The nuclear proteins were extracted from nuclei of intact cells and then incubated with ^{32}P-labeled oligonucleotides encompassing the AP-1 consensus motif (5′-TTC CGG CTG ACT CAT CAA GCG-3′). Figure 3 shows the difference between the coal samples from the Utah and Pennsylvania coal mines in induction of AP-1 binding to consensus target DNA in HTE cells (second band on the gel). The coal from the Pennsylvania coal mine, with a high acid-soluble Fe^{2+} content, activated AP-1 to the same extent as 10 μM H_2O_2. In contrast, the coal from the Utah coal mine without acid-soluble Fe^{2+} did not activate AP-1. The uppermost band on the gel showed the same intensity of the nonspecific binding in lanes B, C, D, and E, indicating that the loading of the nuclear proteins was the same among the four samples. The lowest band originated from the free excess AP-1 probe. These results suggest that the coal from Pennsylvania can upregulate the expression of AP-1 in order to protect cells from oxidant attack.

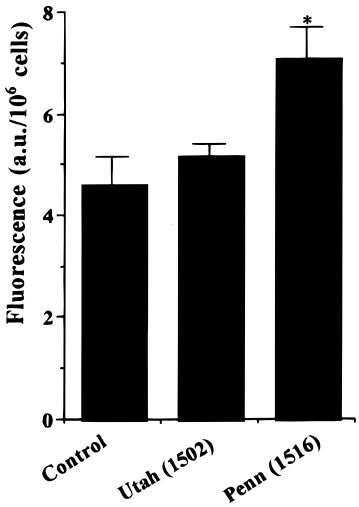

Figure 2. Effect of the two coals from Pennsylvania and Utah coal mines on the formation of oxidants in intact HTE cells as detected by dichlorofluorecein. a.u., arbitrary units; *, significantly different from control by Student's *t*-test, $p < 0.05$.

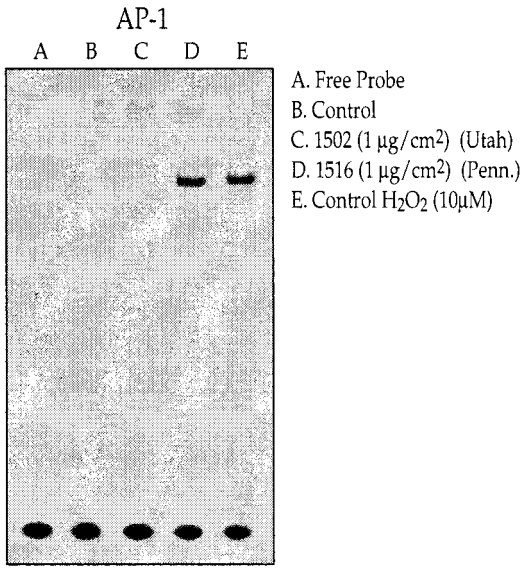

Figure 3. Effect of two coal dusts from the Pennsylvania and Utah coal mines on the activity of AP-1 in HTE cells: (A) free AP-1 probe without nuclear extract; (B) AP-1 probe incubated with nuclear extract from control HTE cells; (C) AP-1 probe incubated with nuclear extract from HTE cells treated with the Utah coal no. 1502 for 5 h; (D) HTE cells treated with the Pennsylvania coal no. 1516 for 5 h; (E) HTE cells treated with 10 μM H_2O_2 for 5 h.

Discussion

CWP is widely believed to be related to oxidative stress. Oxidative stress is a disturbance in the oxidant/antioxi-

dant steady state in favor of oxidants, which leads to cellular damage. A considerable amount of literature has been devoted to the study of ROS in relation to lung injury (*26*).

It is known that Fe^{2+}, in aqueous medium, is able to produce oxidants through Fenton/Haber−Weiss reactions and autoxidation. The former allows for conversion of H_2O_2 and $O_2^{-\bullet}$ into the more reactive $OH^\bullet$ radical according to the following reactions:

$$Fe^{2+} + O_2^{-\bullet} + 2H^+ \rightarrow Fe^{3+} + H_2O_2 \quad (3)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet \quad (4)$$

Interestingly, H_2O_2 and $O_2^{-\bullet}$ may also be produced directly from dissolved oxygen in aqueous medium in an Fe^{2+}-mediated autoxidation reaction:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-\bullet} \quad (5)$$

with subsequent production of H_2O_2 and $OH^\bullet$ according to eqs 3 and 4. Using electron spin resonance and the spin-trapping agent DMPO, we have shown that Fe^{2+} is able to reduce O_2 in an aqueous medium (O_2 solubility in water: 3%, v/v) to produce oxidants (Table 2). Undoubtedly, the abundance of O_2 and H_2O_2 in the pulmonary medium can greatly facilitate the production of $OH^\bullet$ after inhalation of Fe^{2+}-containing particles, which results in lung injury (*27*, *28*). To our knowledge, the pulmonary fibrogenicity of Fe^{2+} compounds in experimental animals has not yet been tested.

On the basis of our observations, we can postulate that Fe^{2+} in coal dust is probably the active compound which induces CWP, while the buffering capacity of the coal is the most important factor in controlling the bioavailability of Fe^{2+}. First, buffering capacity plays a crucial role in the formation and stability of Fe^{2+} in coal dusts. We have previously shown that high buffering capacity cannot stabilize $FeSO_4$, which results from pyrite oxidation (*24*). Second, buffering capacity can probably control, as shown in the acidification assay of the present study, the release of Fe^{2+} in the phagolysosomes of macrophages following phagocytosis. Obviously, it also controls the release in macrophages of other metals such as nickel, arsenic, lead, and cadmium, which are present in trace amounts in coals. Since Fe^{2+} is the most efficient transition-metal ions in yielding oxidants in aerated aqueous medium, and iron along with silica and aluminum represent the most abundant metals in coals, associating the high CWP prevalence with acid-soluble Fe^{2+} in the coal was not an arbitrary choice in the present study.

In the present study, we have shown that buffering capacity is related to the presence of calcite ($CaCO_3$) in the coals (Figure 1). Calcite can easily react with H_2SO_4 to produce $CaSO_4$, which is much less harmful than $FeSO_4$. We observed that coals which were shown to induce high prevalence of CWP contain much less calcite. Thus, these coals have low buffering capacity, and Fe^{2+} in these coals can be easily released in macrophages. In contrast, coals which were shown to induce low prevalence of CWP contain more calcite. For example, the coal of Gardanne from Provence, France, which contained a large amount of $CaCO_3$ had a very high buffering capacity (3340 μL of 1 M H_2SO_4 needed to drop the pH of the aqueous coal suspension to 4.5). This coal did not release any detectable Fe^{2+} in 50 mM HCl. Interestingly, no CWP was reported in the miners who worked in this Gardanne coal mine (*9*). It has been shown by epidemiological studies that the coals from Utah were less hazardous to coal workers than the coals from Pennsylvania and West Virginia. Our studies have shown that Utah coals had a higher buffering capacity and lower acid-soluble Fe^{2+} content than the coals from the east coast, supporting our hypothesis. These results suggest that calcite may play an antagonistic role in CWP development by preventing the solubilization of Fe^{2+}.

In the present study, we observed that all the samples from Pennsylvania coal mines, but not those from West Virginia and Utah, had Fe^{2+} in distilled water (data not shown). Since the coal samples were prewashed with distilled water, the data presented in Table 1 reveals only the acid-soluble Fe^{2+} content. The water-soluble Fe^{2+} may play a role in the emphysema of coal workers, because this part of Fe^{2+} can quickly dissolve in lung medium and become unphagocytosable by AMs. The samples from West Virginia coal mines did not release as much Fe^{2+} at pH 4.5 as we had expected. Under our experimental conditions (10 mM phosphate solution, pH 4.5, for 3 h of incubation), we only measured the Fe^{2+} released most probably from $FeCO_3$ and possibly from $FeSiO_3$ in the coals. Another source of Fe^{2+} is the oxidation of pyrite, a typical component of coal. Pyrite is insoluble in water but can be oxidized to form $FeSO_4$ according to the following reaction 6:

$$2FeS_2 + 7O_2 + 2H_2O \rightarrow 2FeSO_4 + 2H_2SO_4 \quad (6)$$

We have previously shown that factors such as oxygen partial pressure, temperature, and relative humidity played a role in the reaction (*24*). It can take at least a few days to be able to observe the formation of $FeSO_4$. Again, the stability of the formed Fe^{2+} depends on the buffering capacity of the coal. Since we have shown that the buffering capacities of the samples from West Virginia coal mines were low, we can expect that these coals will not neutralize the H_2SO_4 produced by pyrite oxidation or the acid available in the phagolysosomes of macrophages. Therefore, these coal samples can gradually release Fe^{2+} in aqueous suspensions through pyrite oxidation. This factor has not been taken into account in the present study, and may result in an underestimation of bioavailable Fe^{2+} in both the West Virginia and Pennsylvania coal samples. However, it awaits further investigation.

Following dissolution, Fe^{2+} becomes available for oxidant formation within cells as shown by the fluorescence assay (Figure 2). The formed oxidant may consequently activate the antioxidative system of cells. We have demonstrated that low-buffering-capacity coal containing acid-soluble Fe^{2+} enhanced the expression of oxidative stress-responsive transcription factor AP-1 (Figure 3). It is known that transcription factors play a central role in converting extracellular signals into changes in the expression of specific genes and thereby regulate complex biological processes. AP-1 is characterized by its ability to alter gene expression in response to growth factors and cytokines (*29*). Increasing evidence demonstrates that the development and progression of simple pneumoconiosis and progressive massive fibrosis are related to the secretion by macrophages of numerous factors, which enhance fibroblast growth and stimulate the production

of collagen by fibroblasts (*16*). Interleukin-1, macrophage-derived growth factor, tumor necrosis factor-α, and fibronectin are factors that have been shown to stimulate fibroblast growth (*16*). However, component(s) of coal dusts that induce the secretion of growth factors and cytokines is not yet known. Our results suggest that acid-soluble Fe^{2+} is the active component of coal dust which induces cytokine release. The differences in acid-soluble Fe^{2+} content and buffering capacity among coals may explain why CWP is so irregularly distributed among coal workers from different coal mines.

It is noteworthy that the acid-soluble Fe^{2+} and buffering capacity of coals did not systematically increase or decrease with time. Since the first round of the National Study of CWP, followup studies have revealed that, although the prevalence of CWP and dust exposures on the whole have been reduced, the regional differences in CWP have persisted. The highest risk of incurring the disease is associated with Pennsylvania and West Virginia coal mines, whereas the lowest risk is associated with Utah and Colorado coal mines (*13*). This is consistent with our observation of the persistence of acid-soluble Fe^{2+} content and buffering capacity of coals over time, further strengthening our hypothesis that these two parameters may be responsible for the observed regional differences in the prevalence of CWP.

In support of our hypothesis on the role of Fe^{2+} in CWP, pulmonary injury after aspiration of $FeSO_4$ has been reported in a patient showing acute bronchial damage and early histological change in the biopsy specimens after the exposure (*30*). A delayed occurrence of bronchial stenosis after inhalation of iron tablets has also been described (*31*, *32*). In addition, primary cultures of rabbit tracheal epithelial cells were treated with different mineral particles containing Fe^{2+}. Among the tested particles (i.e., chrysotile, nemalite, and hematite), the most cytostatic after 24 h of treatment was nemalite, which had the most Fe^{2+} available on its surface and which produced the most ROS (*33*). This effect of nemalite on cell survival and production of ROS was reduced by pretreatment with desferoxamine, a specific iron chelator. Other reports have also suggested that the generation of ROS by Fe^{2+} may be an important factor in the pulmonary damage induced by asbestos as well as natural and synthetic mineral fibers (*34*).

In conclusion, acid-soluble Fe^{2+} in coal dust may be the active component responsible for inducing CWP through oxidative stress mechanisms. The bioavailability of Fe^{2+} is controlled by the buffering capacity of coal, which is primarily related to the presence of calcite. However, a large number of coal samples from mines with known prevalences of CWP are needed to validate our findings. Studies on these samples will allow us to characterize the distribution of Fe^{2+} and $CaCO_3$ in the coals of different U.S. geographic locations and to establish a database that may be useful for the assessment of coal dust-induced risk of CWP. To our knowledge, an adequate statistical database does not yet exist to determine the average buffering capacity and levels of acid-soluble Fe^{2+} in the coals of different U.S. coal mines. These two parameters are worth considering if we want to predict which coals will lead to a higher incidence of CWP.

Acknowledgment. This project was supported by the National Institute for Occupational Safety and Health Grant OH 03253 (X.H.) and Center Grant ES00260 from the National Institute of Environmental Health Sciences. The expert assistance of Ximei Jin in measurements of buffering capacity and Fe^{2+} in coals and Ilona Jaspers in AP-1 assays is gratefully acknowledged.

References

(1) Miller, B. G., and Jacobsen, M. (1985) Dust exposure, pneumoconiosis and mortality of coal miners. *Br. J. Ind. Med.* **42**, 723−733.

(2) Attfield, M., and Wagner, G. (1992) Respiratory disease in coal miners. In *Environmental and Occupational Medicine*, 2nd ed. (Rom, W., Ed.) pp 325−344, Lillte, Brown and Company, Boston.

(3) Hejny, H., Robock, K., and Armbruster, L. (1989) On the question of quartz evaluation in mine dusts from coal mining and its specific harmfulness. In *Results of studies on dust suppression and silicosis prevention in hard coal mining* (Steinkohlenbergbauverein, Hauptsezernat Staubbekamfung, Eds.) Vol. 17, pp 139−158, Franz-Fisher-Weg 61, D4300 Essen 13.

(4) Walton, W. H., Dodgson, J., Hadden, G. G., and Jacobsen, M. (1977) The effect of quartz and other noncoal dusts in coal workers' pneumoconiosis. In *Inhaled Particles IV* (Walton, W. H., Ed.) pp 669−689, Pergamon Press, Oxoford.

(5) Gough, J. (1940) Pneumoconiosis in coal trimmers. *J. Pathol. Bacteriol.* **51**, 277−285.

(6) Lister, W. B., and Winborne, D. (1972) Carbon pneumoconiosis in a synthetic graphite workers. *Br. J. Ind. Med.* **29**, 108−110.

(7) Attfield, M. D., and Castellan, R. M. (1992) Epidemiological data on US coal miners's pneumoniosis, 1960−1988. *Am. J. Public Health* **82**, 964−970.

(8) Hurley, J. F., Burns, J., Copland, L., et al. (1982) Coal workers' simple pneumoconiosis and exposure to dust at 10 British coal mines. *Br. J. Ind. Med.* **39**, 120−127.

(9) Amoudru, C. (1987) Pneumoconiosis − the actual dimensions of problems. In *Silicosis and mixed-dusts pneumoconiosis* (Le Bouffant, L., Ed.) Vol. 155, pp 3−39, Colloque INSERM, Paris.

(10) Reisner, M. T. R., and Robock, K. (1977) Results of epidemiological, mineralogical and cytotoxicological studies on the pathogenicity of coal mine dusts. In *Inhaled Particles* (Walton, W. H., Ed.) pp 703−715, Pergamon Press, Oxoford.

(11) Morgan, W. K. C., Burgess, D. B., Jacobson, G., O'Brien, R. J., Pendergrass, E., Reger, R. B., and Shoub, E. P. (1973) The prevalence of coal workers' pneumoconiosis in US coal miners. *Arch. Environ. Health* **27**, 221−230.

(12) Attfield, M. D., and Morring, K. (1992) An investigation into the relationship between coal workers' pneumoconiosis and dust exposure in US coal miners. *Am. Ind. Hyg. Assoc. J.* **53**, 248−255.

(13) Attfield, M. D., and Seixas, N. S. (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**, 137−157.

(14) Crawford, N. P., Bodsworth, F. L., and Dodgson, J. (1982) A study of apparent anomalies between dust levels and pneumoconiosis at several British collieries. *Ann. Occup. Hyg.* **26**, 725−744.

(15) Meyers, R. A. (1982) *Coal Structure*, Academic Press, New York.

(16) Lapp, N. L., and Castranova, V. (1993) How silicosis and coal workers pneumoconiosis develop- a cellular assessment. *Occup. Med.* **8**, 35−56.

(17) Marafante, E., Lundborg, M., Vahter, M., and Camner, P. (1986) Dissolution of two arsenic compounds by alveolar macrophages. *Fundam. Appl. Toxicol.* **8**, 382−389.

(18) Lundborg, M., Eklund, A., Lind, B., and Camner, P. (1985) Dissolution of metals by human and rabbit macrophages. *Br. J. Ind. Med.* **42**, 642−645.

(19) Kreyling, W. G., Nyberg, K., Nolibe, D., Collier, C. G., Camner, P., Heilmann, P., Lirsac, P. N., Lundborg, M., and Mateijkova, E. (1991) Interspecies comparison of phagolysosomal pH in alveolar macrophages. *Inhal. Toxicol.* **3**, 91−100.

(20) Huang, X., Laurent, P. A., Zalma, R., and Pezerat, H. (1993a) Inactivation of alpha-1-antitrypsin by aqueous coal solutions. Possible relation to the emphysema of coal workers. *Chem. Res. Toxicol.* **6**, 450−458.

(21) Huang, X., Frenkel, K., Klein, C. B., and Costa, M. (1993b) Nickel induces increased oxidants in intact cultured mammalian cells as detected by dichlorofluorescein fluorescence. *Toxicol. Appl. Pharmacol.* **120**, 29−36.

(22) Salnikow, K., Gao, M., Voitkun, V., Huang, X., and Costa, M. (1994) Altered oxidative stress responses in nickel-resistant mammalian cells. *Cancer Res.* **54**, 6407−6412.

(23) Lainhart, W. S. (1969) Roentgenographic evidence of coal workers' pneumoconiosis in three geographic areas in the United States. *J. Occup. Med.* **11**, 399−408.

(24) Huang, X., Zalma, R., and Pezerat, H. (1994) Factors that influence the formation and stability of FeSO4 in coal dust. Possible relation to the emphysema of coal workers. *Chem. Res. Toxicol.* **7**, 451−459.

(25) Taniguchi, N., Pickett, C. B., and Griffith, O. W. (1993) Oxy radicals and antioxidative responses in cancer: 12th Sapporo cancer seminar. *Cancer Res.* **53**, 3207−3210.

(26) Oxygen radicals and lung injury. *Environ. Health Pespect.* **102** (Suppl. 10) (1994).

(27) Dalal, N. S., Newman, J., Pack, D., Leonard, S., and Vallyathan, V. (1995) Hydroxyl radical generation by coal mine dust: possible implication to coal workers' pneumoconiosis. *Free Radical Biol. Med.* **18**, 11−20.

(28) Chen, L. C., Huang, X., Wu, C. Y., Zalma, R., Pezerat, H., and Qu, Q. S. (1996) The role of FeSO4 in coal dust-induced lung injury. *Appl. Occup. Environ. Hyg.* **11**, 973−979.

(29) Angel, P., and Karin, M. (1991) The role of Jun, Fos, and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta* **1072**, 129−157.

(30) Godden, D. J., Kerr, K. M., Watt, S. J., and Legge, J. S. (1991) Iron lung: bronchoscopic and pathological consequences of aspiration of ferrous sulfate. *Thorax* **46**, 142−143.

(31) Mizuki, M., Onizuka, O., Aoki, T., and Tsuda, T. (1989) A case of remarkable bronchial stenosis due to aspiration of delayed release iron tablet. *Nippon Kyobu Shikkan Gakkai Zasshi* **27**, 234−239.

(32) Tarkka, M., Anttila, S., and Sutinen, S. (1988) Bronchial stenosis after aspiration of an iron tablet. *Chest* **93**, 439−441.

(33) Guilianelli, C., Baeza-Squiban, A., Boisieux, E., et al. (1993) Effect of mineral particles containing iron on primary cultures of rabbit tracheal epithelial cells: Possible implication of oxidative stress. *Environ. Health Perspect.* **101**, 436−442.

(34) Kamp, D. W., Graceffa, P., Pryor, W. A., and Weitzman, S. A. (1992) The role of free radicals in asbestos-induced diseases. *Free Radical Biol. Med.* **12**, 293−315.

TX970151O