Laboratory Studies of the Impact of Calcite on In Vitro and In Vivo Effects of Coal Dust: A Potential Preventive Agent for Coal Workers' Pneumoconiosis?

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Background *Bioavailable iron (BAI) in coal, which may play a key role in causing coal workers' pneumoconiosis (CWP), is present at relatively high levels in Appalachian coals. Calcite decreases BAI and is more plentiful in Western coals than in Appalachian coals, possibly explaining the lower CWP prevalence among Western miners.*

Methods *We measured effects of calcite on BAI in non-cellular and cellular systems involving Pennsylvania (PA) coal dust. We also tested in vivo effects of calcite on transferrin receptor and markers of epithelial mesenchymal transition (EMT) and inflammation in mice exposed to PA coal.*

Results *Calcite rapidly eliminated BAI in an aqueous suspension of PA coal. Ferritin induction in human lung epithelial cells exposed to PA coal was effectively eliminated by calcite. Mouse lung tissue markers indicated increased EMT after exposure to PA coal dust, but not after exposure to PA coal plus calcite. Markers of inflammation increased following exposure to PA coal alone, but not following exposure to PA coal plus calcite.*

Conclusion *Additional research may lead to the use of supplemental calcite in coal mining as a safe and effective way to prevent CWP among Appalachian coal miners.*

KEY WORDS: iron; calcium; coal workers' pneumoconiosis (CWP); epithelial mesenchymal transition (EMT); fibrosis

INTRODUCTION

Coal workers' pneumoconiosis (CWP) was one of the most common occupational diseases in the 20th century. The passage of the Coal Mine Health and Safety Act in 1969 resulted in reduction of occupational exposures to coal mine dust and, thus, decreased incidence of the disease [Weeks, 1993]. Nevertheless, since 2000, the decreas-

Abbreviations: α-SMA, alpha-smooth muscle actin; BAI, bioavailable iron; CWP, coal workers' pneumoconiosis; DAPI, 4′,6-diamidino-2-phenylindole; EMT, epithelial mesenchymal transition; FBS, fetal bovine serum; IL, interleukin; MEM, Modified Eagle's Medium; TfR1, transferrin receptor-1.

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ing trend has reversed and the prevalence of CWP among examined miners with 15 or more years of coal mining tenure has recently increased, particularly in central Appalachia, including coal mining regions of Kentucky (KA), Virginia (VA), and West Virginia (WV) [NIOSH, 2008; Laney and Attfield, 2010; Laney et al., 2010]. Results of previous studies relating to differences in bioavailable iron (BAI) and calcite content between Appalachian coals and Western coals suggest that the constituents of coal may play key roles in the pathogenesis of CWP—BAI increasing coal dust toxicity and calcite, by decreasing BAI, possibly decreasing coal dust toxicity [Huang, 2011]. Thus, while efforts to limit underground miner exposure to coal mine dust must continue, further research is warranted to explore the potential use of calcite as a novel BAI-targeted strategy for preventing CWP.

We have previously shown that BAI in Appalachian coals is one of the factors responsible for lipid peroxidation and alteration of signal transduction and gene expression in cells and inflammation in mice, and that the differing level of BAI in various coals is correlated with observed regional differences in the prevalence of CWP [Huang and Finkelman, 2008; McCunney et al., 2009]. BAI originates from oxidation of pyrite (FeS_2), a typical component of coal dusts. The stability of BAI in aqueous solution is pH-dependent [Singer and Stumm, 1969]. At pH <4.5, BAI is stable (e.g., half-life of 3 years at pH 3.0); at pH >4.5, BAI is precipitated (e.g., half-life of 8 min at pH 7.0). In addition to pH, the stability of BAI in coal dust depends upon how long the coal dust is exposed to air [Huang et al., 1994]. Western coals containing high levels of calcite ($CaCO_3$) have high pH and, consequently, low BAI and less toxicity as measured by lipid peroxidation. Perhaps this calcite-mediated lower toxicity accounts for the relatively low CWP prevalence observed among examined miners at underground Western coal mines [Huang et al., 2005]. Based on CaO levels in high-temperature ashes of Utah (UT) coals, estimated calcite levels in coals from UT are 2–5% (w/w) [Huang et al., 1998; Zhang et al., 2002]. Despite the presence of iron in UT coals, calcite prevents iron in the coals from becoming bioavailable [Huang et al., 1998]. In contrast, Appalachian coals do not contain calcite and have acidic pH and high levels of BAI, which increases lipid peroxidation in cells. Interestingly, CWP prevalence is relatively high among underground Appalachian coal miners. If BAI is indeed a key determinant of the toxicity of coal mine dust responsible for CWP, all the above findings suggest the possibility that calcite might find a place in a novel strategy for preventing CWP among miners exposed to dust from coal containing relatively high levels of BAI. In the present study, we have tested the ability of supplemental calcite to decrease BAI in non-cellular and cellular systems involving Pennsylvania (PA) coal dust and to prevent coal dust effects on transferrin receptor-1 (TfR1) and markers of epithelial mesenchymal transition (EMT) and inflammation in mice exposed to PA coal.

MATERIALS AND METHODS

Cell Line and Reagents

Normal human immortalized bronchial epithelial Beas-2B cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). They were grown in monolayer cultured in Modified Eagle's Medium (MEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Cells were maintained at 5% CO_2, 37°C and 95% humidity and starved in 1% FBS 24 hr prior to dust exposure. All coal samples were purchased from the Penn State Coal Sample Bank; physico-chemical characteristics such as BAI levels, quartz content, coal rank, etc. were previously reported [Huang et al., 1998; Zhang et al., 2002]. Calcite with a particle size of approximately 100 μm (Sigma, St. Louis, MO) was ground to respirable size (<2.5 μm) before experiments. Antibody against mouse ferritin was from Rockland Immunochemicals Inc. (Gilbertsville, PA).

Characterization of Respirable Coal Dusts by X-Ray Fluorescence Spectroscopy

Respirable coal dusts (<2.5 μm) from Pennsylvania (PA, PSOC#1313) and Utah (UT, PSOC#433) coal mine regions were collected on Teflon filters (Millipore, Bedford, MA). Concentrations of iron, calcium, aluminum and sulfur were quantified using X-ray fluorescence spectroscopy (Ex-6600 AF, Jordan Valley, UK).

Elimination of BAI by Calcite in Water

An initial stock solution of calcite was prepared in water and then diluted serially down to the respective concentrations of 10%, 5%, 2%, and 1% with one PA coal (PSOC# 1512) containing an initial BAI level of 1,598 ppm. The mixtures were suspended in water at room temperature at a concentration of 90 mg coal per 1 ml water. A small fraction of the suspension (<0.1 ml) was taken at 5, 10, 20, and 30 min of incubation, respectively, and was filtered through a 0.65-μm MF-Millipore™ membrane filters (Millipore). BAI in the aqueous coal filtrates was detected by a ferrozine-based colorimetric assay (Sigma), monitored spectrophotometrically at 560 nm using a UV–Vis microplate reader (SpectraMax® Plus, Molecular Devices, Sunnyvale, CA).

Immunofluorescence Staining and Confocal Microscopy

To test whether calcite can eliminate BAI in cells, immunofluorescence technique was used to visualize ferritin, an iron storage protein with a capacity of binding up to 4,500 atoms of iron per molecule of ferritin [Koorts and Viljoen, 2007]. In brief, Beas-2B cells were seeded on a cover slip in a 24-well plate and pretreated with the aqueous suspension of BAI-containing PA coal dust, followed by calcite (10% PA coal, w/w) 4 hr later. Untreated Beas-2B cells served as controls. As comparisons, Beas-2B cells were also treated with PA coal dust alone, UT coal dust, calcite, and PA leachate (filtrate from the PA coal suspension). After 2 days, the cells were washed with PBS and fixed in 3.7% formaldehyde for 5 min. The cells were permeabilized with 0.1% Triton-100 for 5 min, and then primed with mouse anti-ferritin antibody (red) to stain ferritin and 4′,6-diamidino-2-phenylindole (DAPI, blue) to label cell nuclei. After washing, the cover slip was mounted on a slide with mounting medium and sealed with nail oil. The samples were imaged by using a Leica TCS SP5 confocal fluorescence microscopy (Mannheim, Germany).

Animal Exposure

All animal experiments were performed according to the protocol approved by the Institutional Animal Care and Use Committee at the New York University School of Medicine. To investigate whether calcite quenches BAI in the PA coal in vivo, 48 male 129/SvTac mice at age 9–11 weeks (Taconic, Hudson, NY) were randomly assigned to four groups. The mice were aspirated with 50 μl of (1) water (control); (2) PA coal (50 μg/50 μl) suspension; (3) calcite (5 μg/50 μl or 10% calcite w/w); and (4) a mixture of PA coal and calcite (45 μg coal and 5 μg calcite/50 μl). Water, rather than saline, was used for the aspiration exposures because our neutralization system is in water and buffered saline at pH 7.4 inactivates BAI prior to aspiration. A subgroup of six mice from each group was sacrificed at 24 and 72 hr, respectively. The right and left lobes of the lungs were collected for RNA analyses.

RNA Isolation and qPCR

RNAs from lung tissues exposed to various dusts were extracted in TRIzol® following the manufacturer's instructions (Invitrogen, Carlsbad, CA). In brief, lung tissues from each mouse were homogenized in 1 ml TRIzol and then 10 μg RNA from each homogenate was pooled to represent the average expressions of each subgroup. For each treatment subgroup, 2 μg pooled RNA were reverse-transcribed by SuperScript® III Reverse Transcriptase (Invitrogen). The cDNA, used as template for qPCR, was mixed with SYBR® Green Supermix (Bio-Rad, Hercules, CA) and the primers of the target genes. The sequences of the primers are listed in Table I. The qPCR was run in a 384-well plate (ABI 7900 series; Applied Biosystems, Foster City, CA). The mRNA expression levels of the target genes were normalized to the geometric mean of three housekeeping genes—GAPDH, G6PD, and HPRT1. Data were expressed as fold changes over the control mice.

RESULTS

Characterization of Respirable Coal Dusts by X-Ray Fluorescence Spectroscopy

Table II shows substantial differences in the elemental composition of the PA coal (PSOC#1313) and the UT coal (PSOC #433). Fluorescence intensities indicate that mineral content (total of Ca, Fe, S, and Al) is greater in the respirable UT coal dust than in the respirable PA coal dust. Notably, calcium level is much higher in the UT coal than in the PA coal, resulting in a ratio of calcium to iron of 3.7 in the UT coal and 0.38 in the PA coal. The finding of a much higher calcium-to-iron ratio in UT coal

TABLE I. Primers for qPCR Analyses of Iron, Epithelial Mesenchymal Transition (EMT), and Inflammatory Markers in Lung Tissue of Mice

Gene name	Gene ID	Forward primer	Reverse primer
TfR1	22042	5′-CATGAGGGAAATCAATGATCG-3′	5′-ACATAGGGCGACAGGAAGTG-3′
E-cadherin	12550	5′-CCAATCCTGATGAAATTGGA-3′	5′-CGAACACCAACAGAGAGTCG-3′
α-SMA	11475	5′-TTGCTGACAGGATGCAGAAG-3′	5′-GTTCTGGAGGGGCAATGAT-3′
IL-1α	16175	5′-AGCGCTCAAGGAGAAGACC-3′	5′-TTTTGGTGTTTCTGGCAACT-3′
IL-1β	16176	5′-GGGCCTCAAAGGAAAGAATC-3′	5′-TATTGCTTGGATCCACACT-3′
GAPDH	14433	5′-GGCATTGCTCTCAATGACAA-3′	5′-CCCTGTTGCTGTAGCCGTAT-3′
HPRT1	15452	5′-TGTTGTTGGATATGCCCTTG-3′	5′-GGCTTTGTATTTGGCTTTTCC-3′
G6PD	14381	5′-CACCACTGCTGCACAAGATT-3′	5′-TCAGCTCATCTGCCTCTGTG-3′

TABLE II. Characterization of Coal Dusts by X-ray Fluorescence (XRF)

Fluorescence intensity[a]	Fe	Ca	S	Al	Ca/Fe ratio
PA [a.u.(%)]	61.5 (24%)	23.3 (9%)	104.3 (40%)	78.9 (28%)	0.38
UT [a.u.(%)]	222.4 (18%)	817.1 (68%)	76.1 (6%)	91.0 (8%)	3.7

[a]XRF data demonstrates the relative amounts as arbitrary units (a.u.) provided by XRF and percentage distributions of major elements in coals from PA (PSOC# 1313) and UT (PSOC #433) coal mine regions.

suggested the possibility that UT coal may be less toxic than PA coal as a result of the calcium preventing the iron from becoming bioavailable. Because calcium in the UT coal is mainly in the form of calcite [Huang et al., 1998], we next added pure calcite to PA coal at various proportions to determine whether and how rapidly calcite can eliminate BAI in the PA coal.

Effects of Added Calcite on BAI in Water Suspensions of PA Coal Dust

As shown in Figure 1, BAI in a water suspension of PA coal was most effectively quenched by 10% calcite. At this calcite concentration, there was a sharp decrease in BAI within the first 5 min and a continual decrease until BAI was nearly totally eliminated by 20 min. Calcite at 5% resulted in an 8% decrease from the initial amount of BAI by 30 min. Adding calcite at lower concentrations (2%, 1%, and 0%) caused no detectable decrease in BAI concentration at 30 min.

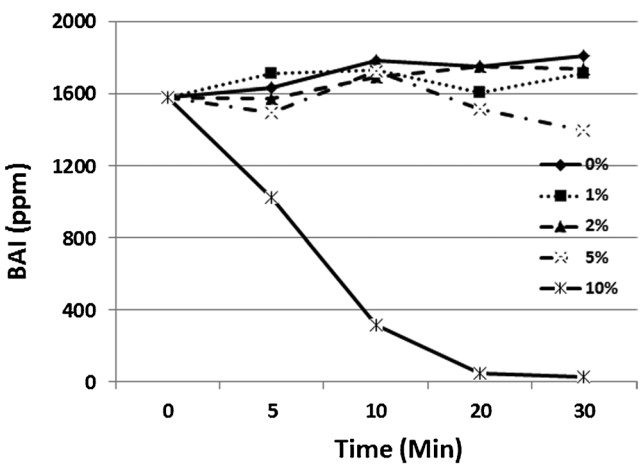

FIGURE 1. Effects of added calcite on BAI in water suspensions of PA coal dust. A water suspension of dust from a PA coal (PSOC# 1512) with an initial BAI level of 1,598 ppm was mixed with 0%, 1%, 2%, 5%, and 10% (w/w) calcite ($CaCO_3$), respectively. After filtration, the iron content in the aqueous coal filtrates was detected by the ferrozine assay.

In Vitro Effects of Calcite on Ferritin in Human Lung Epithelial Cells Treated With Various Coal Dusts and Calcite

We tested the effect on intracellular BAI of the addition of calcite to Beas-2B cells 4 hr post coal dust exposure. Figure 2 shows that control Beas-2B cells contain ferritin. Treatment with PA coal dust alone and leachate extracted from the PA coal substantially induced ferritin in these cells. In contrast, treatment with UT coal dust or pure calcite did not induce ferritin. Most notably, the addition of calcite 4 hr post PA coal dust exposure completely attenuated induction of ferritin by the PA coal.

In Vivo Effects of Calcite on Regulation of Transferrin Receptor-1 (TfR1)

qPCR was used to analyze changes in iron metabolism in lung tissues of the control and exposed mice. It is well established that, when iron levels are high in cells, TfR1, a membrane receptor controlling iron uptake, is down-regulated [Ganz, 2008; Hentze et al., 2010]. Indeed, at 24 hr post-exposure, mRNA levels of TfR1 in mice exposed to the BAI-containing PA coal dust were down-regulated by 44% (Fig. 3). Notably, calcite alone substantially up-regulated TfR1 mRNA levels (sevenfold increase compared to control), as did the mixture of PA coal dust with calcite (ninefold increase). At 72 hr post-exposure, mRNA levels of TfR1 were back to control levels in all groups (data not shown).

In Vivo Effects of Calcite on PA Coal Dust-Induced Markers of EMT and Inflammation

It is well established that conventional testing of animals shows no clear effects of coal dust on fibrosis [Castranova and Vallyathan, 2000]. However, increasing evidence demonstrates that EMT caused by tissue injury leads to fibrosis [Kalluri and Neilson, 2003; Kalluri, 2009]. To determine whether EMT is caused by coal dust exposure, we measured mRNA levels of EMT markers in the lung tissues of mice exposed by aspiration to PA coal dust alone, calcite alone, or a mixture of both. Figure 4

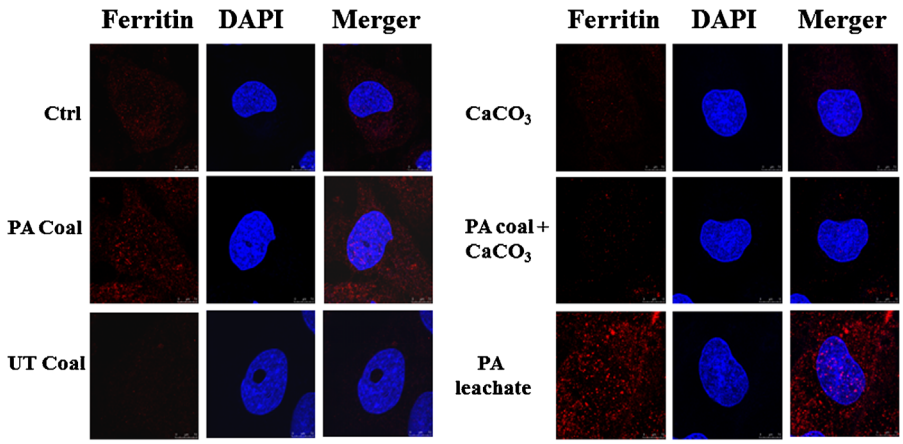

FIGURE 2. Levels of ferritin in human lung epithelial Beas-2B cells treated with various coal dusts and calcite. Red shows ferritin in cells and blue shows cell nuclei. Merger is the combination of the two views from two different excitation/emission wavelengths for red and blue fluorescence.

shows that at 24 hr post-exposure, the BAI-containing PA coal dust down-regulated epithelial marker E-cadherin mRNA and up-regulated fibroblastic marker alpha-smooth muscle actin (α-SMA) mRNA. The PA coal dust also increased mRNA expression for the inflammatory cytokines interleukin-1alpha (IL-1α) and interleukin-1beta (IL-1β). Interestingly, the mixture of the PA coal with calcite prevented the PA coal dust-induced EMT markers by significantly increasing E-cadherin and decreasing α-SMA relative to the effects of PA coal dust alone. The mixture also inhibited the PA coal dust-induced inflammation markers of IL-1α, and IL-1β. Calcite alone significantly increased IL-1α and IL-1β, suggesting an inflammatory response in mice after exposure to calcite alone. At 72 hr post-exposure, mRNA levels of all four markers returned to control levels in all three treatment groups (data not shown).

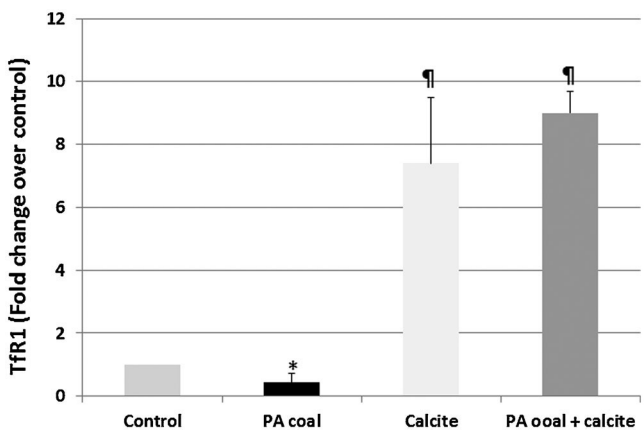

FIGURE 3. Effects of calcite on regulation of transferrin receptor-1 (TfR1) in vivo. In the presence of high iron, TfR1 is down-regulated in lung tissue of mice exposed to the BAI-containing PA coal dust (n = 6). Calcite alone or a mixture of the PA coal with calcite up-regulates TfR1 (3rd and 4th bars). *: Significantly different from control mice exposed to water ($P < 0.05$, n = 6); ¶: Significantly different from mice exposed to PA coal dust ($P < 0.05$, n = 6).

DISCUSSION

The World Coal Institute estimates that proven coal reserves will last at least 120 years at current production levels of approximately 5 billion tons per year (http://www.worldcoal.org). Thus, occupational health problems associated with exposure to coal mine dust are likely to persist into the foreseeable future unless exposures are more effectively controlled. Health and environmental costs, including occupational lung disease compensation, have been estimated to increase the cost of energy from a new coal-fired power plant by 60–100% [Jacobson and Masters, 2001]. Hence, reducing CWP occurrence could financially benefit society and the coal industry.

In coal mining, calcite is widely used as the main constituent of rock dust that is applied in underground coal mines for prevention of dust explosions and in the treatment of acid mine drainage. Pulverized calcite is added to the boilers of coal-fired power plants to absorb SO_2 and NO_x, important components of the air pollution that causes acid rain. Given these existing uses, introduction of additional calcite into coal before or during mining should not cause substantial environmental concern, and may even have beneficial effects on acid mine drainage.

Calcium is an essential human nutrient required in substantial amounts. Dietary calcium recommendation was recently increased from 800 to 1,200 mg/day for persons aged >51 years old [Straub, 2007]. In nutrition, calcite (calcium carbonate) is the most common calcium supplement because it has the highest concentration of calcium

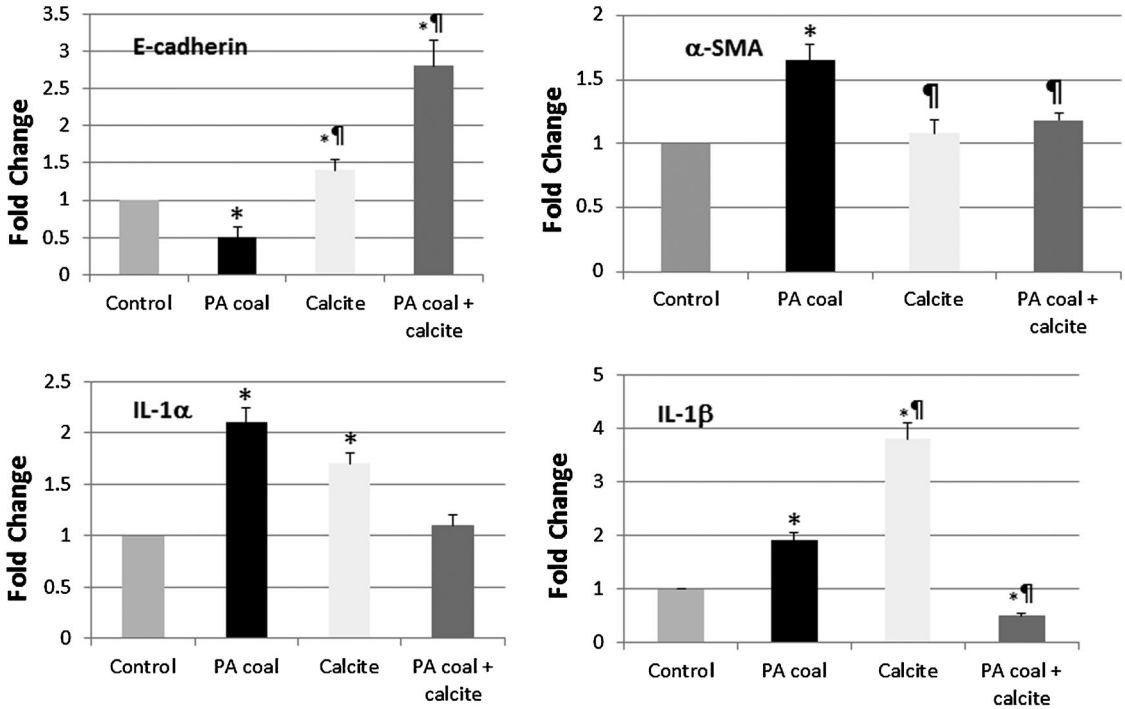

FIGURE 4. Changes in markers of EMT and inflammation as measured by qPCR in mice exposed to BAI-containing PA coal dust, calcite, or the mixture of PA coal and calcite. E-cadherin and α-SMA are used as markers of EMT for epithelial and fibroblast cells. IL-1α and IL-1β are used as markers of inflammation. *: Significantly different from control mice exposed to water ($P < 0.05$, n = 6); ¶: Significantly different from mice exposed to the PA coal dust ($P < 0.05$, n = 6).

by weight (40%), as compared to other common calcium supplements (e.g., calcium citrate, acetate, phosphate, and gluconate). Calcium carbonate is also commonly taken orally as an over-the-counter treatment for heartburn and acid indigestion. It has long been known that orally administered calcium decrease iron absorption and, thus, BAI [Cook et al., 1991; Prather and Miller, 1992].

Despite its presence at relatively high levels in Western coals, its wide use in coal mining, and its common use as an antacid and nutritional supplement, the safety and effectiveness of any proposed use of calcite as a potential preventive agent for CWP needs to be addressed. In the present study, we examined both calcite's ability to prevent adverse effects induced by PA coal dust and whether a small amount of calcite along with Appalachian coal might do less harm than either alone.

We found that the ratio of calcium to iron content is higher in respirable dust from less toxic UT coal than in respirable dust from more toxic PA coal (Table II). For various coals, respirable-size dust has iron and calcium composition similar to bulk samples of the same coals [Huang et al., 1998, 2005]. These findings suggest that, compared to Western coal miners, Appalachian coal miners inhale dust with a much lower calcium-to-iron ratio.

We have previously shown that elimination of BAI by addition of calcite to a suspension of PA coal dust in a buffered solution mimicking phagolysosomal fluid could take weeks [Zhang and Huang, 2005]. In the present study, we demonstrated that calcite is able to eliminate BAI relatively rapidly in an aqueous suspension of PA coal dust; calcite at 10% w/w nearly completely eliminated 1,600 ppm BAI within 20 min (Fig. 1). These results support our notion that BAI in coal dust might be substantially reduced by adding calcite into water sprays used in coal mining for dust control. The contact time between sprayed calcite and airborne respirable dust may not be sufficient to entirely eliminate BAI, but elimination of BAI by calcite may continue to occur after the dust is deposited in the lung. To examine whether calcite can still reduce BAI even hours after lung epithelial cells are exposed to coal dust, we found that calcite treatment 4 hr after PA coal exposure effectively decreased levels of ferritin in Beas-2B cells (Fig. 2). Calcite alone also reduced endogenous levels of intracellular ferritin. These results demonstrate the versatility of calcite in removing BAI in the coal dust as well as in the cells. Silica is considered a toxic component of mixed coal dust. It has been shown by others that endogenous ferritin is an important source of iron which can be sequestered by silica, leading to oxidative stress

[Fubini et al., 2001]. This finding suggests that calcite might be able to prevent toxicity caused by exposure to crystalline silica. Thus, experiments similar to those carried out in the present study should be done to assess the potential for calcite to prevent adverse effects induced by silica dust.

CWP is a fibrotic disease associated with chronic inflammation caused by the inhalation of respirable coal dust [Chong et al., 2006]. In animal studies, inflammatory responses are often observed following coal dust inhalation [Castranova and Vallyathan, 2000; Huang and Finkelman, 2008]. Because current methods for assessing CWP (e.g., X-ray, MRI, etc.) lack sufficient sensitivity and coal dusts are not as strongly fibrogenic as quartz or bleomycin in mice [Degryse and Lawson, 2011], fibrotic stages of CWP have never been reported in mice. To overcome this difficulty, we measured mRNA expression of EMT markers as a more sensitive and earlier indicator of the disease process.

The role of EMT caused by tissue injury in leading to eventual fibrosis is becoming increasingly apparent [Kalluri and Neilson, 2003; Kalluri and Weinberg, 2009]. More specifically, EMT is found to be associated with progressive fibrosis occurring in kidney, lung, liver, and intestine [Kim et al., 2006; Zeisberg et al., 2007a,b]. Direct evidence indicates that epithelial cells serve, via EMT, as important precursors of fibroblasts that arise during the course of fibrosis [Strutz et al., 1995; Okada et al., 1997]. E-cadherin is a marker of epithelial cells and α-SMA is a marker of mesenchymal and fibroblast cells. These markers have provided reliable signatures to characterize the transition from epithelial to mesenchymal to fibroblast cells that occurs during development of fibrosis [Okada et al., 1997; Zeisberg et al., 2007a,b]. Changes in these markers in lung tissue provided one of the first indications that epithelial cells under oxidative and inflammatory stress can transition through EMT to fibroblasts [Strutz et al., 1995], ultimately shedding all their epithelial markers and gaining a fully fibroblastic phenotype. In the present study, we found that aspiration exposure of mice to PA coal dust, which contains relatively high levels of BAI, decreased mRNA levels of E-cadherin and increased mRNA levels of α-SMA in lung tissue, suggesting a transition of epithelial cells to fibroblasts. More importantly, when exposure was to a mixture of PA coal dust with calcite, the observed levels of TfR1 and altered EMT markers pointed to a biological reaction involving a BAI-eliminating non-fibrotic process (Figs. 3 and 4). Although calcite alone caused an apparent inflammatory response, we showed that aspiration of a mixture of PA coal dust with a small amount of calcite resulted in less inflammatory response than either alone.

Taken together, results of our study indicate that (1) calcite is able to eliminate BAI in coal dust and (2) in the presence of calcite, BAI-containing coal dust appears to be non-fibrogenic. Thus, calcite holds promise for reducing the incidence of CWP among Appalachian coal miners. How a calcite-based preventive technology might be implemented needs further investigation. In a mining setting, two potential technologies to introduce calcite into coal mines could be explored. First, it may be possible to introduce respirable calcite during mining through a second water spray system. The primary water spray currently in use is to wet the airborne dust particles and makes the contacted particles larger and heavier, causing them to fall out of the air (i.e., they are no longer respirable). A second water spray system with respirable calcite may be used to target the escaped respirable coal particles. Future research should assess whether adding calcite to these water sprays can be done safely and effectively, with the intent of not only reducing dust levels but also neutralizing adverse health effects of BAI contained in the respirable coal dust.

A second potential calcite-based technology might be especially applicable in Appalachian coal fields, which have much higher methane content than other US coal fields. To help prevent methane explosions during mining, many coal mining companies have employed technologies that degasify the coal bed and recover methane before coal is extracted. The hydraulic fracturing process used to degasify coal beds might be an ideal time to introduce calcite to the coal seam. A predetermined amount of calcite (e.g., 20–30 mesh size) could be added along with sand to the fracturing water to serve as a proppant to keep the coal seam fractures open. Degas holes normally have a service time ranging from a few months to 2 years or even longer before the degassed coal is extracted. Assuming diffusion of the calcite suspension throughout the coal seam, this would allow sufficient time for the injected calcite to neutralize the BAI in the coal. If such an approach proves feasible, the toxicity of dust produced in cutting the calcite-treated coal might be greatly reduced. Yet, much preliminary research should be done in a lab before attempting any large-scale calcite injection in coal seams.

Lastly, whether calcite can be used as an inhalant to prevent or treat CWP is another potential option that needs to be examined. Calcite neutralizes acid, producing nontoxic calcium ions and carbon dioxide. However, the safety of inhaling calcite remains unknown. Would the alkalinity of calcite prove problematic in the lungs? Can it be buffered, or would that negate its' effect on neutralizing BAI? Our study shows that changes in EMT markers in mice provides unprecedented opportunities to study the pathogenesis and prevention of CWP by identifying very early effects of dust and potential preventive agents (such as calcite) on gene regulation. Thus, these safety concerns and effectiveness in CWP prevention can be answered through further laboratory investigations.

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