Induction of interleukin-6 by coal containing bioavailable iron is through both hydroxyl radical and ferryl species

Q ZHANG and X HUANG*

New York University School of Medicine, Department of Environmental Medicine, 550 First Avenue, PHL Room 802, New York, NY 10016, USA

**Corresponding author (Fax, 212-263-6649; Email, xihuang@env.med.nyu.edu)*

Coal mining causes health problems, such as pneumoconiosis. We have previously shown that prevalence of pneumoconiosis in workers from various coalmine regions positively correlates with levels of bioavailable iron (BAI) in the coals from that region. In the present study, the nature of reactive oxygen species formed by BAI in the coals and its mechanisms of the induction of biological responses were investigated. Human lung epithelial cell line, A549 cells, were used to examine the induction of interleukin-6 (IL-6), a pro-inflammatory cytokine, which is known to play a crucial role in the development of pneumoconiosis. We found that levels of IL-6 protein as well as its mRNA were significantly increased in the cells treated for 24 h with 20 μg/cm^2 of the BAI-containing Pennsylvania (PA) coal; for example we observed 6·7–fold increase in IL-6 protein. Levels of IL-6 protein in cells treated with the Utah (UT) coal containing low-BAI were only 1·9-fold of the control levels. The enhancing effect on the IL-6 by the PA coal was similar to that caused by hydrogen peroxide. Superoxide dismutase (SOD), catalase (CAT), and N-acetyl-L-cysteine (NAC) all had inhibitory effects on the PA coal-induced IL-6 formation. However, CAT had the least protective effect as compared to SOD and NAC. Our results indicate that BAI in the PA coal may induce IL-6 through both ferryl species (via iron autoxidation) and hydroxyl radicals (via the Fenton/Haber Weiss reactions).

[Zhang Q and Huang X 2003 Induction of interleukin-6 by coal containing bioavailable iron is through both hydroxyl radical and ferryl species; *J. Biosci*. **28** 95–100]

1. Introduction

Epidemiological studies of the relationship between the prevalence of coal workers' pneumoconiosis (CWP) and environmental measurements have consistently revealed that the predominant adverse exposure factor is respirable mixed coal dusts (Attfield and Wagner 1993). It has been shown in the US that the disease is more prevalent in Pennsylvania (PA) coal miners and least common in coal miners from Utah (UT) (Attfield and Castellan 1992; Morgan *et al* 1973). Chronic obstructive pulmonary diseases, such as asthma and emphysema, indistinguishable from their non-occupational analogues, also appear to be more prevalent in PA coal workers than in UT coal workers. Our previous studies have indicated that the prevalence of CWP among various coalmine regions positively correlates with average levels of bioavailable iron (BAI) in the coals from that region (Huang *et al* 1998; Zhang *et al* 2002). We have further shown that coals from the PA coalmine regions with a high prevalence of CWP induce large amounts of ferritin in cells.

Keywords. Coal; interleukin-6; iron; oxidative stress; pneumoconiosis

Abbreviations used: BAI, Bioavailable iron; CAT, catalase; CWP, coal workers' pneumoconiosis; HO$^{\cdot}$, hydroxyl radical; α-MEM, α-minimum essential medium; NAC, N-acetyl-L-cysteine; ($O_2^{\cdot-}$), superoxide anion; PA, Pennsylvania; SOD, superoxide dismutase; UT, Utah.

Coals from West Virginia (WV), with an intermediate prevalence of CWP, induce moderate levels of ferritin. In contrast, levels of ferritin in cells treated with the coals from UT, with a low prevalence of CWP, were only marginally increased as compared to the control levels (Zhang and Huang 2002).

Iron is a well-known transition metal capable of catalyzing oxidant formation by interacting with oxygen (O_2) and/or hydrogen peroxide (H_2O_2). CWP is long considered as one of the human lung pathologies related to oxidative stress (Castranova and Vallyathan 2000). Therefore, BAI in the coals may play an important role in coal dust-induced CWP through oxidative stress pathways. Fenton and Haber-Weiss reactions are generally accepted mechanisms for participation of iron in biological free radical oxidation (Toyokuni 1996). These reactions initially require the presence of H_2O_2, which results in the hydroxyl radical ($HO^{\bullet}$) formation. However, in biological systems, with the high physiological ratio of $[O_2]/[H_2O_2]$ ($\geq 10^3$), the nature of oxidant species responsible for the BAI-containing coal-induced biological responses is not well known. Because of the abundance of O_2 in aqueous media, ferrous ion autoxidation, which requires only Fe^{2+} and O_2, may be an important route to the initiation of detrimental biological free radical oxidations (Qian and Buettner 1999). This reaction would result in the formation of iron–oxygen (Fe–O) complexes, such as perferryl or ferryl ions, with high electron affinity, initiating oxidation. Therefore, knowing the exact nature of oxidants formed by BAI in the coal may provide important information for clinical treatment of the disease. The goal of the present study was to determine the oxidant species catalyzed by BAI in coal-induced oxidative stress, which may contribute to cell injury *in vitro*, and possibly lead to the development of CWP. IL-6 is an inflammatory cytokine that can be both mitogenic (involved in cell proliferation) and fibrogenic (involved in extracellular matrix synthesis) (Kayano and Okita 2000; Kelley 1990; Suganuma *et al* 2002). Using IL-6 as a biomarker of cell injury, we have studied the ability of coals from PA and UT coalmine regions to induce IL-6 as well as its mRNA in human lung epithelial A549 cells. Because lung epithelial cells contain proteases which can clear fibrin deposits and repair tissues, effects of coals on these cells determine the ultimate degree of permanent lung damage (Lee *et al* 1994). Using various antioxidants and enzymes, we have evaluated the pathways leading to the oxidant formation and subsequent cell injury by the BAI-containing coals. Our results suggest that both ferryl species and hydroxyl radicals may be responsible for initiating the process of oxidative damage. Therefore, simple use of one antioxidant may not be sufficiently effective for the prevention of cell injury.

2. Materials and methods

2.1 *Cell culture*

Human lung epithelial cell line, A549, with characteristics of alveolar epithelial type II cells (ATCC CCL185), was used for our experiments. Cells were maintained in α -minimum essential medium (α-MEM) completed with 10% FBS, 1% antibiotics and 2 mM L-glutamine and grown in 95% air, 5% CO_2 at 37ºC.

2.2 *Selection and preparation of coal samples for cell treatment*

Samples of coal from PA (high-CWP area) and UT (low-CWP area) were used. They were purchased from the Penn State Coal Sample Bank (Pennsylvania State University, PA, USA). Coal samples were size-classified using the Mercer Impactor (Zhang *et al* 2002). Coal particles less than 5 μm were used for cell treatment.

2.3 *Cell treatments*

Human lung epithelial A549 cells were grown in complete α-MEM until 60–70% confluence. Before treatment, medium was changed to α-MEM completed with 1% serum and incubated overnight. It was found that 1% serum gave maximal induction of IL-6 after coal treatment (data not shown). Cells were incubated with coals from PA and UT for 24 h at a single dose of 20 $\mu g/cm^2$, which produced high levels of IL-6 protein with above 80% survival rate. Non-treated cells were used as controls. To assess the nature of oxidants catalyzed by BAI in the PA coal, cells were pretreated with superoxide dismutase (SOD), catalase (CAT), or N-acetyl-L-cysteine (NAC) for 1 h, followed by the addition of freshly prepared PA coal suspension. H_2O_2 was used as a positive control for these experiments. After treatment, cell culture media were collected and stored at – 80ºC for IL-6 determination using ELISA kit (R&D Systems). Cells were washed and scraped for RNA isolation.

2.4 *RNA isolation and RT-PCR*

Total RNA was extracted using Trizol solution. Reverse transcription was carried out using Superscript RNase H^- reverse transcriptase according to the manufacturer's instructions. Mouse IL-6 fragments were amplified by 30 cycles of PCR, each cycle consisting of 90 s at 94ºC, 45 s at 58ºC and 90 s at 72ºC. All PCR reactions were carried out in the linear range of IL-6. The resultant RT-PCR products along with DNA markers were electrophoresed

in 2% agarose gels, then stained with 1X SYBR and visualized under UV light. The RT-PCR bands were analysed by densitometric scanning (Imaging Densitometer, model GS-700; Bio-Rad Laboratories) using the molecular Analyst Software. For semi-quantitative analysis, mRNA of the housekeeping gene GAPDH was co-amplified using the same RT-PCR conditions as for each experiment. Ratios of all experimental bands to GAPDH were used to measure changes in mRNA expression between different treatment groups.

2.5 *Statistics*

Student's *t* test with two-tails and equal variances was used for statistical significance calculation. Data have been presented as the average of three independent experiments ± standard error (SE).

3. Results

3.1 *Effects of PA and UT coals on the IL-6 formation*

A549 cells were treated with one PA coal (Psoc# 337) and one UT coal (Psoc# 500). Table 1 shows some physico-chemical characteristics of coals that may be important in the development of CWP. For example, levels of silica content and coal rank as estimated by the carbon content or molar ratio of carbon/hydrogen (C/H) were comparable between the two coals. In contrast, the coal from the PA coalmine region contained a large amount of BAI (both Fe^{2+} and Fe^{3+}), while the coal from UT had considerably less. Interestingly, at 20 μg/cm^2, levels of IL-6 dramatically increased in cells treated with PA coal (6·7-fold of control), but to a much lesser extent, in cells treated with the UT coal (1·9-fold) as compared to the control levels (124·7 ± 11·1 pg/ml). Using H_2O_2 as a positive control, we found that in 24 h 0·25 mM of H_2O_2 increased IL-6 in A549 cells by 2·9-fold to 366·8 ± 12·7 pg/ml, with a further increase by 0·5 mM and 1 mM H_2O_2 of 4·4-fold and 3·9-fold, respectively.

3.2 *Effects of SOD, CAT, and NAC on the PA coal-induced IL-6 and its mRNA*

Figure 1 shows the inhibitory effects of SOD, an enzyme decomposing superoxide anion ($O_2^{\cdot-}$), CAT, an enzyme decomposing H_2O_2; and NAC, a general antioxidant, on the levels of IL-6 induced by the PA coal or H_2O_2. As shown in the figure 1, 300 units SOD or CAT/ml cell culture media or 10 mM NAC all inhibited, to large extent, the PA coal- or H_2O_2-induced IL-6. Increasing the

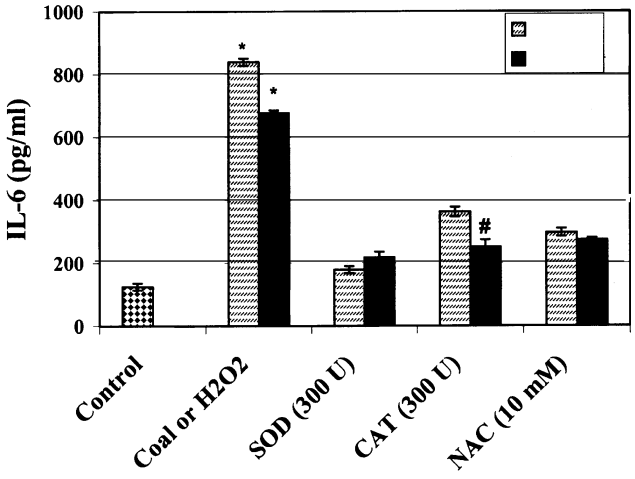

Figure 1. Induction of IL-6 by the PA coal (20 μg/cm^2) or H_2O_2 (0·5 mM) and the inhibitory effects of SOD, CAT, and NAC on the PA coal- and H_2O_2-induced IL-6 in human lung epithelial A549 cells. *, Significantly different from the control by Student's *t* test ($P < 0·05$); #, significantly different from the PA coal + CAT by Student's *t* test ($P < 0·05$).

Table 1. Physico-chemical characteristics of PA and UT coal samples and the induction of IL-6 protein in human lung epithelial A549 cells by the coals at 20 μg/cm^2.

Coals	Carbon[a]	Atomic C/H molar ratio[a]	Silica[a]	Bioavailable iron (ppm)[b] Fe^{2+}	Bioavailable iron (ppm)[b] Fe^{3+}	IL-6 (pg/ml)[c]
PA (Psoc# 337)	56·5%	1·22	0·45%	2786·4	7244·1	838·9 ± 11·4
UT (Psoc# 500)	46·8%	1·10	0·54%	19·3	25·8	237·4 ± 46·7

[a], Data on carbon content or molar ratio of carbon/hydrogen as an indicator of coal rank and silica content in the coals were provided by the Penn State Coal Sample Bank and Database.
[b], Levels of bioavailable iron (both Fe^{2+} and Fe^{3+}) were determined spectrophotometrically by 2,2-dipyrydil-Fe^{2+} and deferoxamine-Fe^{3+} complexes at 520 nm and 430 nm, respectively. Bioavailable iron is defined as iron released in 10 mM phosphate solution, pH 4·5, which mimics the phagolysosomes of cells.
[c], IL-6 was determined using ELISA kit from R&D System and was expressed as pg/ml cell culture media. Levels of IL-6 in the medium of control A549 cells were 124·7 ± 11·1 pg/ml.

concentrations of SOD or CAT up to 8000 U/ml did not improve the inhibitory effects of these enzymes on IL-6 formation. Interestingly, the inhibitory effect of CAT on H_2O_2-induced IL-6 was significantly ($P < 0{\cdot}05$, $n = 3$) more pronounced (63·0% inhibition) than on the BAI-containing PA coal-induced IL-6 (53·9% inhibition). Figure 2 shows a higher induction of IL-6 mRNA expression by the PA coal dust treatment than the coal from UT at 20 μg/cm^2 and similar effect was induced by H_2O_2. SOD and CAT inhibited IL-6 mRNA expression (figure 2). Again, the inhibitory effects of SOD on the PA coal and H_2O_2–induced IL-6 mRNA were comparable, while the inhibitory effect of CAT on H_2O_2-induced IL-6 mRNA was more pronounced than on the BAI-containing PA coal-induced IL-6 mRNA.

4. Discussion

Increasing evidence demonstrates that CWP is one of the human lung pathologies related to oxidative stress and chronic inflammation (Castranova and Vallyathan 2000). For example, previous studies on symptomatic coal miners have shown that alveolar macrophages, recovered from broncho-alveolar lavage, released excessive amounts of oxidants and inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and IL-6 (Rom 1990, 1991; Vallyathan *et al* 2000; Vanhee *et al* 1995). Our previous studies have shown that BAI may be responsible for the oxidant formation in cells treated with BAI-containing coals and contribute to the subsequent transactivation of nuclear factor of activated T cells (NFAT) and activator protein-1 (AP-1) (Huang *et al* 1998, 1999, 2002a). In the present study, we have shown that the coal from the PA coalmine region with a high level of BAI significantly increases the levels of IL-6 in human lung epithelial A549 cells. The coal from UT with a low level of BAI also increases the levels of IL-6, but to a lesser extent, as compared to the PA coal. To support the role of BAI in the induction of IL-6, treatment of cells with pure ferrous sulphate also significantly increased IL-6 protein (manuscript in preparation). Therefore, differences in the levels of BAI in the coals may be responsible for the differences in the IL-6 induction, and thus, contribute to

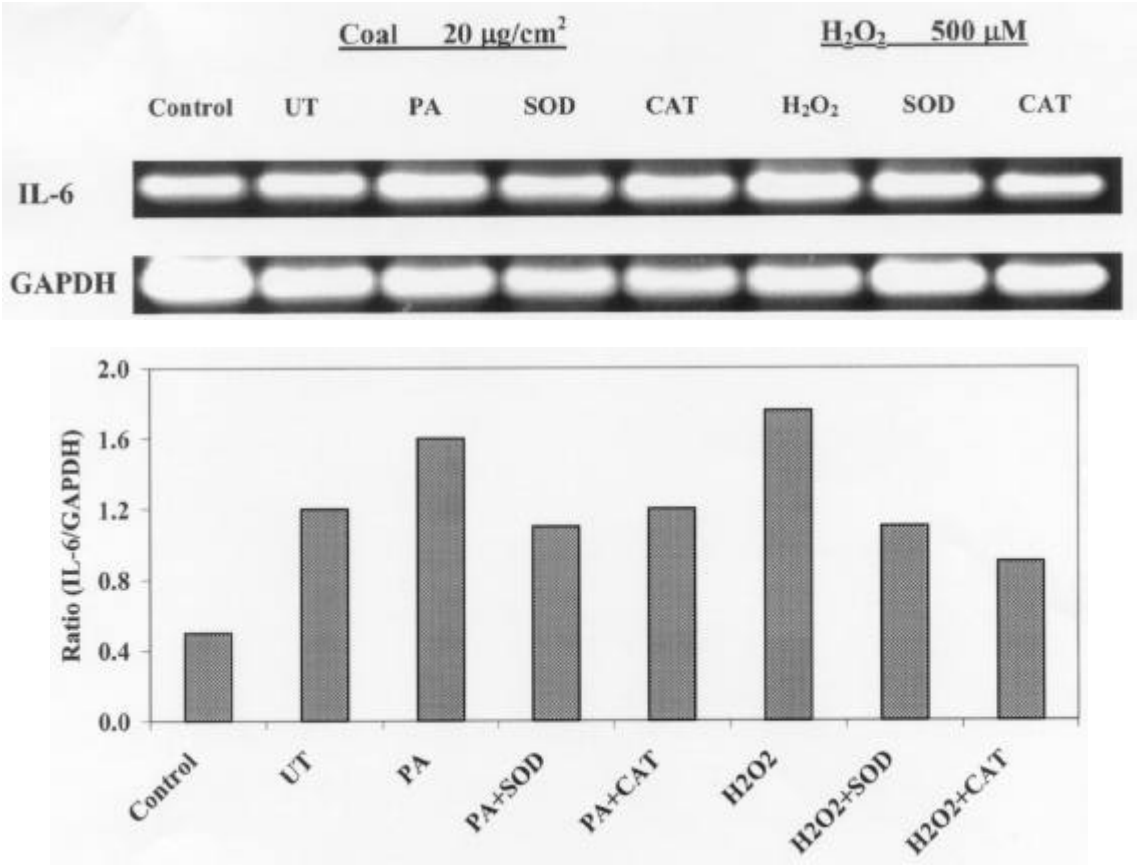

Figure 2. Induction of IL-6 mRNA by the PA coal or H_2O_2 and the preventive effects of SOD and CAT on the PA coal- and H_2O_2-induced IL-6 mRNA in human lung epithelial A549 cells.

the observed regional differences in the prevalence of CWP.

IL-6 is involved in cell activation, growth, and differentiation. Alteration of IL-6 gene may play an important role in a variety of diseases, including CWP (Kelley 1990; Kayano and Okita 2000; Vallyathan *et al* 2000; Wedzicha 2002). IL-6 promoter region contains several transcription factor binding sites, such as AP-1 and nuclear factor–κB (NF–κB), which are responsive to oxidative stress (Beetz *et al* 2000; Kosmidou *et al* 2002). Our previous studies have shown that the PA coals with BAI can transactivate AP-1 in mouse epidermal cells and in human A549 cells (Huang *et al* 2002a). The UT coal was not as active as the PA coal in inducing AP-1 (Huang *et al* 2002a) and IL-6, shown by the present study. Therefore, the UT coal was not further tested with NAC, SOD or CAT. These results suggest that increased levels of IL-6 in cells treated with the PA coal may be through the AP-1 activation pathways following BAI-induced oxidative stress. However, the oxidant species formed by the BAI in cells are not well characterized. Knowing the nature of oxidants may lead to better strategies for disease prevention and clinical intervention.

In biological systems, it is often considered that oxidants formed by iron originate from the interaction of iron with enzymatically and/or non-enzymatically generated superoxide ($O_2^{\cdot-}$) (Haber-Weiss reaction) and/or hydrogen peroxide (H_2O_2) (Fenton reaction) as illustrated below:

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{\text{Iron}} O_2 + OH^- + HO^{\bullet} \quad \text{(Haber-Weiss reaction)} \quad (1)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + HO^{\bullet} \quad \text{(Fenton reaction)} \quad (2)$$

It is noteworthy that Fenton and Haber-Weiss reactions require the presence of H_2O_2 (Eqs 1, 2) to produce $HO^{\bullet}$. If BAI present in the PA coals induce IL-6 through these two reactions, one would expect that CAT should completely inhibit the PA coal-induced IL-6. However, the inhibitory effect of CAT was less than that of SOD and NAC, even in the case of H_2O_2 treatment. These results suggest that reactions other than Fenton and Haber-Weiss may be involved in the BAI-containing PA coal-induced IL-6.

In fact, an alternate mechanism has been previously proposed in a cell-free system (Qian and Buettner 1999). Free radical oxidations, such as lipid peroxidation, are not necessarily initiated by $HO^{\bullet}$ formed from the Fenton or Haber-Weiss reactions, but rather, oxidations are initiated by iron in the form of 'Fe–O' complexes, such as perferryl or ferryl ions (Qian and Buettner 1999; Huang *et al* 2002b). Measurements in cells have determined the steady state level of H_2O_2 to be approximately 10^{-8} M (Boveris and Cadenas 1997), and the steady state level of O_2 *in vivo* is about 10^{-5} M (Jones 1986), three orders of magnitude higher. Assuming that the rate constant for oxidation of substrate by '$Fe^{2+} + O_2$' chemistry (Fe^{2+} autoxidation) is similar to Fenton reaction and that the oxidizable substrate concentration in a living system is about 1 M, it has been estimated that the rate of oxidation of oxidizable substrate by '$Fe^{2+} + O_2$' could be as much as 10^8 faster than the rate of oxidation by the Fenton reaction (Qian and Buettner 1999). Due to their high electrophilic properties, perferryl and ferryl ions are important oxidants in detrimental biological oxidations, with reactivities approaching that of $HO^{\bullet}$.

The perferryl ion is an intermediate product that can be poduced through either Fe^{2+}/O_2 or $Fe^{3+}/O_2^{\cdot-}$ (Eq. 3)

$$Fe^{2+} + O_2 \rightarrow [Fe^{2+}\text{-}O_2 \text{ or } Fe^{3+}\text{-}O_2^{\cdot-}] \leftarrow Fe^{3+} + O_2^{\cdot-} \quad \text{(Perferryl ion)} \quad (3)$$

The ferryl ion can be formed by the reaction of perferryl ion with another Fe^{2+} (Eqs 4 and 5)

$$Fe^{2+} + Fe^{2+}\text{-}O_2 \rightarrow Fe^{2+}\text{-}O_2\text{-}Fe^{2+} \quad (4)$$

$$Fe^{2+} - O_2 - Fe^{2+} \rightarrow 2\ Fe^{2+}\text{–O}. \quad (5)$$

In cells treated with PA coals, Fe^{2+}, Fe^{3+}, O_2 and $O_2^{\cdot-}$ are present, and therefore, are capable of yielding ferryl and perferryl species. Obviously, oxidation initiated by these ferryl and perferryl species cannot be prevented by CAT treatment. As shown in figure 1, the inhibition by SOD and NAC was more effective than CAT on the PA coal-induced IL-6. These results suggest that ferryl species formed via iron autoxidation are equally important as $HO^{\bullet}$ via Fenton/Haber-Weiss reactions in cellular free radical oxidation catalyzed by BAI in the coals.

In summary, our experiments with SOD and CAT clearly demonstrate that $O_2^{\cdot-}$ and H_2O_2 are involved in the oxidation reactions initiated by the BAI present in the PA coal. The resulting $HO^{\bullet}$ from the Fenton and Haber-Weiss reactions may subsequently induce IL-6 in A549 cells. The partial inhibition by the CAT on the PA coal-induced IL-6 suggests that the Fenton reaction could be operative, but it only explains a part of the outcome. The more effective inhibition of IL-6 by SOD and NAC than CAT suggests that perferryl or ferryl ions may be the oxidative species as important as $HO^{\bullet}$ in BAI-containing PA coal-induced IL-6 formation.

Acknowledgements

This project was supported by grant OH 03561 from the National Institute for Occupational Safety and Health, and in part by the NIEHS Center Grant ES00260.

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