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EFFECT OF INHALED CHROMIUM ON PULMONARY A1AT

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A major health hazard to coal miners is development of emphysema following long-term exposure to coal dust. One mechanism underlying development of emphysema is the oxidation of critical methionine (Met) residues in antiproteolytic factor, α1-antitrypsin (A1AT) resulting in a protease–antiprotease imbalance in the lung. Several studies have documented an association between the incidence and severity of emphysema among miners and their exposure to crystalline silica (i.e., SiO_2). However, what remains unclear is the role of other co-inhaled nonemphysematogenic nonoxidant inorganic constituent in disease pathogenesis. We hypothesize that in miners, inhaled trivalent chromium (Cr^{3+}, the only form of Cr in coal) may potentially affect lung A1AT activity in situ via Cr complexing with Met residues, and thereby exacerbate any SiO_2-induced imbalance. To ascertain if Cr^{3+} could, in fact, affect A1AT activity, in vitro studies were done to assess elastase inhibitory activity following A1AT incubation with soluble Cr^{3+}. In addition, to determine if Cr^{3+} found in the lungs as detoxification products of inhaled hexavalent Cr (Cr^{6+}) could affect A1AT in situ, lavages from the lungs of chromate-exposed rats were also analyzed for elastase inhibitory activity The in vitro results indicate that Cr^{3+} ions clearly inhibited A1AT function, with an IC50 of 1.1 mM being estimated under the experimental conditions used. The in vivo results indicate that long-term inhalation (12 wk or longer) of chromate-bearing atmospheres also gave rise to significant (i.e., 50–70%) inhibition of the antielastase activity of A1AT. Together, these results clearly suggest that the Cr^{3+} present in coal dusts could potentially act to inhibit A1AT activity in the lungs of miners and thereby promote the emphysematogenicity of SiO_2 or of other emphysematogens present as coconstituents in these dusts.

Epidemiologic studies have shown that exposure to coal dust is correlated with an increase in the incidence of emphysema (Ryder et al., 1970; Oxman et al., 1993; Leigh et al., 1994; Borm, 1997). In most mining situations, silicas (i.e., SiO_2) are major constituents of the complex particulate mixtures to which miners are exposed (USGS, 1998). Several studies have clearly documented a strong correlation between the incidence/severity of emphysema among miners and their exposure to crystalline silica (i.e., quartz). One mechanism underlying development of emphysema is that inhaled SiO_2

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induces increased elastinolysis of lung connective tissue via a protease–antiprotease imbalance—in part, through oxidation of critical methionine (Met) residues in the lung antiproteolytic factor, α1-antitrypsin (A1AT) (reviewed in Janoff, 1985). That SiO_2 might cause this imbalance in situ is supported by in vitro studies showing that SiO_2-induced reactive oxygen species (ROS) oxidize A1AT Met residues and reduce antielastase activity (Zay et al., 1995; Churg et al., 1997). Although a role for SiO_2 in coal-dust-induced emphysema is clear, what remains unclear is the role of other co-inhaled nonemphysematogenic nonoxidant inorganic constituents in disease pathogenesis or amplification. We hypothesize that in miners, inhaled trivalent chromium (Cr^{3+}, the only form of Cr in coal; Huggins et al., 2000) may potentially affect lung A1AT activity in situ via Cr complexing with Met residues not previously damaged by ROS or repaired by methionine sulfoxide reductase (MsrA), and thereby exacerbate any SiO_2-induced protease–antiprotease imbalance. To directly ascertain whether Cr^{3+} could, in fact, affect A1AT activity, in vitro studies were performed to assess elastase inhibitory activity following A1AT incubation with soluble Cr^{3+}. In addition, to determine whether Cr^{3+} found in the lungs as detoxification products of inhaled hexavalent Cr (Cr^{6+}) could affect A1AT in situ, lavages from Cr^{6+}-exposed rat lungs were analyzed.

MATERIALS AND METHODS

Chromium(III) chloride ($CrCl_3$) and all other reagents used in A1AT analyses were obtained from Sigma (St. Louis, MO). To analyze Cr^{3+} effects on A1AT, equal aliquots of 0.2 mg human A1AT/ml phosphate-buffered saline (PBS) (150 m*M* NaCl/10 m*M* sodium phosphate, pH 7.4) and freshly prepared aqueous $CrCl_3$ solutions of varying strength (0.5–4.0 m*M*) were combined and incubated 24 h at 37°C. To assess A1AT activity, 5 µl PPE (porcine pancreatic elastase, Type III, 50 µg/ml PBS-1% albumin) solution was mixed with 10 µl A1AT test solution and 85 µl PBS and incubated at 37°C for 90 min. The mixture was combined with 400 µl 1.25 m*M* SLAPN [*N*-succinyl-$(Ala)_3$-*p*-nitroanilide]; triplicate 100-µl aliquots were transferred to microtiter plate wells, and absorbances were monitored at 405 nm every 30 min for 2 h. Percent elastase-inhibiting activity was calculated as $\%EIA = 100 \times (E_c - E_{trt})/(E_c - E_{A1AT})$, where E_{trt}, E_{A1AT}, and E_c were elastase activity when Cr-treated, native, or no A1AT, respectively, was present. Control wells had $CrCl_3$ incubated overnight with PBS in place of A1AT ("aged" $CrCl_3$), aged $CrCl_3$ + PPE + SLAPN, or aged $CrCl_3$ + SLAPN to assess potential effects of Cr^{3+} on absorbance, PPE activity, or substrate stability, respectively. As a $CrCl_3$ concentration-dependent effect on pH was noted, studies using A1AT incubated 24 h in PBS of varying pH were also done.

To analyze A1AT from Cr^{6+}-exposed hosts, male F344 rats (Charles River, Raleigh, NC) were exposed (10 rats/exposure group/duration) nose-only, 5 h/day, 5 days/wk for 4, 8, 12, 24, or 48 wk to filtered air or to 360-µg Cr/m^3

aerosols generated by nebulizing aqueous calcium chromate suspensions (pH 7) as previously described (Cohen et al., 2002). Hourly samples were collected on dedicated port filters to monitor Cr levels. Particle size was assessed using a Mercer cascade impactor. Three days after their final exposure, rats were euthanized by Nembutal overdose (120 mg/kg, ip) and their lungs were processed to obtain acellular bronchoalveolar lavage fluid (BALF) (Cohen et al., 1997).

To assess A1AT activity, 50 µl BALF was combined with 50 µl 2 µg PPE/ml solution, placed in a microtitre plate well, and incubated 90 min at 37°C. This PPE concentration was selected based on expected A1AT activity in naive rat BALF (Johnson et al., 1990); use of this particular level would also assure that no elastase should remain unreacted after combination with the BALF so as to potentially mask any exposure-related change in A1AT activity. Following the incubation, 50 µl HEPES with m*M* $CaCl_2$ 0.01% Tween-20, 50 µg/ml albumin solution (pH 7.5) was added. After gentle mixing, 50 µl 1 m*M* SLAPN was added and absorbance was monitored at 405 nm every 15 min for 2 h. The %EIA in each BALF sample was calculated as relative change in elastase activity from that in reactions that contained only PBS (lavage vehicle) in combination with PPE. Based upon the total protein present in each BALF, EIA% values, and a 1:1 A1AT:elastase stoichiometry, total active A1AT/total BALF protein (ng/µg) in each sample was also calculated.

Data were analyzed to determine statistical significance of any differences between exposure groups (at each time point) or within each group (as a function of exposure duration). This was performed using two-way analysis of variance (ANOVA) followed, when appropriate, by a least-squares means test; results were considered significant at $p < .05$.

RESULTS

Absorbance, PPE activity, or SLAPN status was not affected by $CrCl_3$ in any of the in vitro studies. However, $CrCl_3$ did exhibit a narrow dose-dependent inhibitory effect (IC50 of ~1.1 m*M* Cr^{3+}) against A1AT (Figure 1A). When reactions contained <500 µ*M* $CrC1_3$, inhibition of A1AT was minimal; conversely, if Cr^{3+} reached >1.5 m*M*, inhibition was nearly complete. Because of the concern that any effects were an artifact associated with the pH of the A1AT–$CrCl_3$ mixtures, the impact of incubation of A1AT in PBS of varying pH values was evaluated (Figure 1B). Significant A1AT inhibition did not occur until reaction pH was <5.5; complete abrogation of activity was attained at pH 3.5. Because a pH of ≤5.5 was not achieved until $CrCl_3$ concentrations were at least 1.25–1.50 m*M* in the reactions, it was concluded that the loss of A1AT activity as shown in Figure 1A was primarily attributable to the presence of Cr^{3+} in the mixture rather than to the direct influence of pH.

Figure 2A shows that repeated inhalation of Cr-bearing atmospheres [exposure levels were 315–363 µg Cr/m^3 over a 48-wk period; particle size (MMAD) was 0.6 µm ($\sigma_g = 1.7$)] gave rise to changes in activity of A1AT

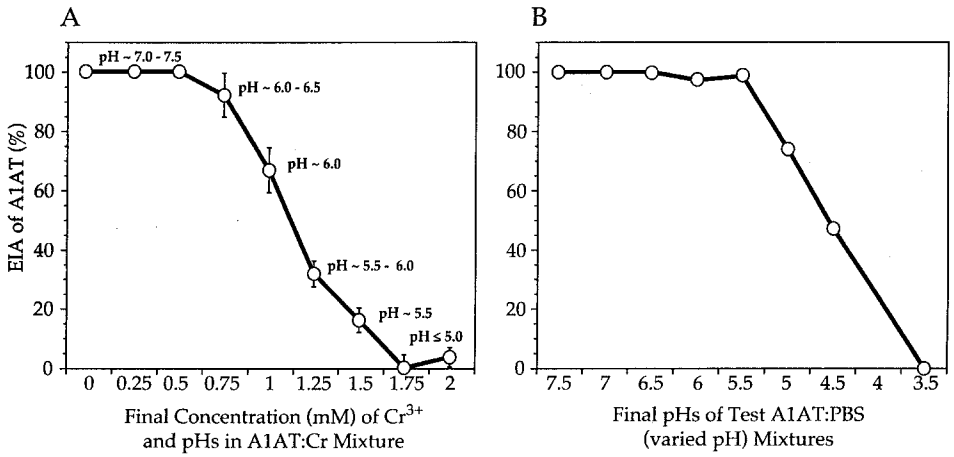

FIGURE 1. Elastase-inhibiting activity (%EIA) of human A1AT after 24-h incubation at 37°C with (A) varying concentrations of $CrCl_3$ or (B) PBS buffers of varying pH that bracket values attained with the $CrCl_3$:A1AT solutions. Remaining inhibitory activity of the A1AT samples were assessed against PPE using SLAPN substrate as outlined in the Methods. Data shown are representative of the %EIA activity calculated after 90-min incubation of the reaction mixtures with SLAPN substrate.

present in lungs of exposed rats. The data indicate that repeated inhalation of Cr for a period of >8 wk resulted in reductions of A1AT activities consistently at 50–70% of control levels. This effect is also reflected as decreases in amounts of active A1AT as a component of all BALF protein (Figure 2B).

DISCUSSION

These studies are the first to demonstrate the ability of Cr^{3+} to affect the activity of α1-antitrypsin (A1AT), the major lung antiproteolytic factor. This finding is critical to enhancing our understanding of potential risks to the health of miners from exposure to coal dusts, in that Cr^{3+} is a common constituent of particulate atmospheres in mines. Recent studies reported average coal Cr content worldwide as 20 ppm; Cr is present at 4–54 ppm (μg/g) in Kentucky coals, although other American coals have >100 ppm Cr (Brownfield et al., 1995; IARC, 1997; Huggins et al., 2000). The Cr^{3+} in coals is localized primarily into organic macerals and mineral (i.e., in clay illite) fractions (Gluskoter et al., 1977; Huggins et al., 2000). The distribution among fractions may be critical to the potential for Cr^{3+} to act as a toxicant, as Cr^{3+} in illite is more easily released than is that in organic matrices. It follows then that miners exposed to coal dusts containing significant amounts of Cr-bearing illite would inspire greater numbers of particles from which Cr^{3+} could ultimately be more bioavailable in the lungs than would miners working maceral-rich seams. Consequently, the former would be at an increased risk from toxicity evolving from the eventual liberation of dust-associated Cr^{3+} in their lungs.

That coal-dust-associated Cr^{3+} may induce effects in situ that could enhance SiO_2-induced emphysematous changes is based on its cross-linking

nature. It is known that Cr^{3+} readily forms complexes with many biomolecules, especially proteins and free amino acids. Certain amino acids preferentially complex with Cr^{3+}. The most common occur among residues that contain sulfur, with the frequency being cysteine > cystine > methionine (Salnikow et al., 1992; Voitkun et al., 1994; Zhitkovich et al., 1995). As

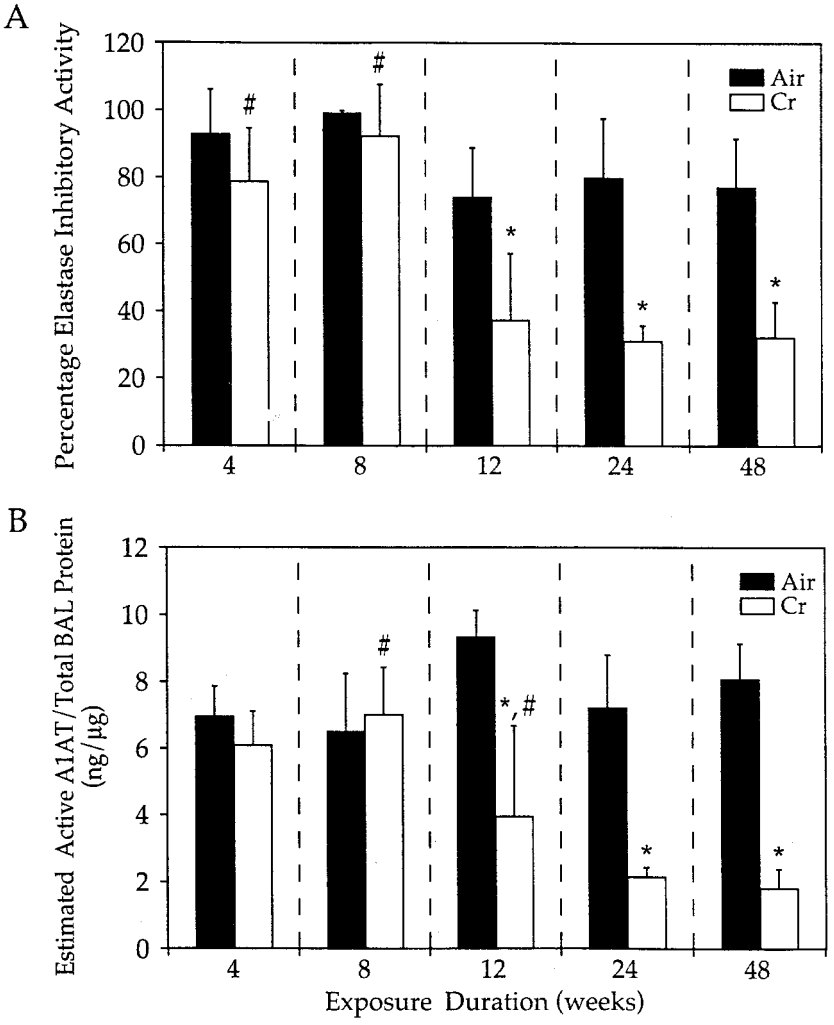

FIGURE 2. (A) Elastase-inhibiting activity (%EIA) of A1AT recovered from the lungs of rats repeatedly exposed to calcium chromate for varying periods of time. Inhibitory activity of the A1AT in recovered BALF samples were assessed against PPE using SLAPN substrate as outlined in the Methods. Data shown represents %EIA activity after 60-min incubation of reaction mixtures with SLAPN. (B) Estimated active A1AT as a component of all proteins found in each BALF sample. In both figures, each bar value represents the mean (± SE) of individual samples from a total of five rats per exposure group. Within an exposure group, values significantly ($^{\#}p < .05$) different from their counterparts at (A) 12, 24, and 48 wk and (B) 24 and 48 wk are indicated. Within a given exposure duration, values significantly different due to exposure regimen are also indicated ($^{*}p < .05$).

such, the possibility exists that the structural integrity of extracellular proteins in the lungs, including that of A1AT, might be affected by inhaled Cr^{3+}.

Under in vitro conditions, $CrCl_3$ induced a narrow dose-dependent inhibitory effect against A1AT. The 1.1 m*M* Cr^{3+} IC50 was similar (albeit stronger) to that noted in coal-dust-induced A1AT inhibition studies focusing on Fe^{2+} (IC50 ~1.75 m*M* Fe^{2+}) (Huang et al., 1993). While a criticism could be made that this Cr^{3+} level is not likely attained in situ, it should be considered that the A1AT concentration used in these in vitro studies was ~100 times greater than that expected in rat or human lungs (i.e., ~1–2 μg/ml) (Johnson et al., 1990). If the stoichiometry of this observed inhibition is maintained within the lungs, then an in situ IC50 of ~11 μ*M* could reasonably be postulated. Achieving this concentration over the total 6 L lung volume of miners (especially those at coal face, where dust levels can average 10 mg/m^3) over a span of >20–30 yr would not be improbable, since Cr^{3+} is well retained (Baetjer et al., 1959). Furthermore, the actual total amount of Cr^{3+} needed to reach this IC50 would be reduced substantially if toxicity was evaluated in terms of localized (i.e., in a few milliliters volume in a subregion of lung) effects observed rather than those over the entire volume of the organ.

To assess if inhibitory effects of Cr^{3+} on A1AT also occurred in situ, it would have been optimal to analyze BALF from hosts exposed to a Cr^{3+}-bearing atmosphere. In this study, rather than initiating de novo exposures, BALF from rats that had been repeatedly exposed to occupationally encountered Cr^{6+} agents for periods up to 48 wk (Cohen et al., 1997, 1998, 2002) were analyzed for A1AT antielastase activity. The justification for this approach was twofold: (1) Cr^{3+} formed in the pulmonary extracellular spaces as detoxification products of inhaled Cr^{6+} would then be able to interact with the extracellular A1AT, and (2) use of BALF from hosts exposed for increasing total periods would permit a preliminary determination to be made as to whether some threshold level of Cr in the lungs needed to be attained for an effect to be observed.

The results reported herein showing that rats exposed to chromate-bearing atmospheres display decreased A1AT antielastase activity are also novel. It is interesting that in rats exposed for 12, 24, or 48 wk, average Cr concentrations in lavages of the entire lung were only 0.25, 0.37, and 0.45 μ*M*, respectively. Because the extent of inhibited A1AT activity was 50–70% of that found in controls at these levels, the true Cr^{3+} IC50 in situ value might be well below that just proposed. In addition, because BALF protein concentrations did not significantly differ between Cr- and air-exposed rats until 48 wk, this precludes simple dilution (i.e., other proteins "crowding out" A1AT) as a basis for our results. Lastly, concurrent analyses of BALF MsrA did not provide any indication that repair enzyme levels were altered in Cr-exposed rats (data not shown); this suggests that the inhibited A1AT activity in Cr-exposed rat BALF was not likely a result of simple oxidation of the critical Met residue. In combination with the in vitro data, this strongly supports

our hypothesis that inhalation of Cr^{3+}-bearing atmospheres (such as coal dust) may potentially affect lung A1AT activity in situ.

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