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Crystalline Silica is a Negative Modifier of Pulmonary Cytochrome P-4501A1 Induction

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Polycyclic aromatic hydrocarbons (PAHs) are products of incomplete combustion that are commonly inhaled by workers in the dusty trades. Many PAHs are metabolized by cytochrome P-4501A1 (CYP1A1), which may facilitate excretion but may activate pulmonary carcinogens. PAHs also stimulate their own metabolism by inducing CYP1A1. Recent studies suggest that respirable coal dust exposure inhibits induction of pulmonary CYP1A1 using the model PAH β-naphthoflavone. The effect of the occupational particulate respirable crystalline silica was investigated on PAH-dependent pulmonary CYP1A1 induction. Male Sprague-Dawley rats were exposed to intratracheal silica or vehicle and then intraperitoneal β-naphthoflavone, a CYP1A1 inducer, and/or phenobarbital, an inducer of hepatic CYP2B1, or vehicle. β-Naphthoflavone induced pulmonary CYP1A1, but silica attenuated this β-naphthoflavone-induced CYP1A1 activity and also suppressed the activity of CYP2B1, the major constituitive CYP in rat lung. The magnitude of CYP activity suppression was similar regardless of silica exposure dose within a range of 5 to 20 mg/rat. Phenobarbital and β-naphthoflavone had no effect on pulmonary CYP2B1 activity. Both enzymatic immunohistochemistry and immunofluorescent staining for CYP1A1 indicated that sites of CYP1A1 induction were nonciliated airway epithelial cells, endothelial cells, and the alveolar septum. Using immunofluorescent colocalization of CYP1A1 with cytokeratin 8, a marker of alveolar type II cells, the proximal alveolar region was the site of both increased alveolar type II cells and decreased proportional CYP1A1 expression in alveolar type II cells. Our findings suggest that in PAH-exposed rat lung, silica is a negative modifier of CYP1A1 induction and CYP2B1 activity.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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Cytochrome P-450s (CYPs) play a key role in metabolizing lipophilic chemicals into more soluble compounds. One of the CYPs, cytochrome P4501A1 (CYP1A1), is responsible for the metabolism of polycyclic aromatic hydrocarbons (PAHs), such as those found in tobacco by-products. Unfortunately, the products of PAH metabolism by CYP1A1, while less lipophilic, often induce DNA adducts and are associated with the initiation of cancer (Newbold & Brookes, 1976; Marshall et al., 1984; Denissenko et al., 1996; Smith et al., 2000; Kondraganti et al., 2003; Mollerup et al., 2006). In addition, exposure to the PAH substrate induces CYP1A1 RNA and protein, resulting in markedly increased metabolic activity and production of more potentially carcinogenic metabolites of the PAH substrate (Denison & Whitlock, 1995).

For workers in the dusty trades, both particles and PAHs may be in the workplace air, producing a mixed exposure. One of the occupational dust exposures of concern is respirable crystalline silica, which was classified as a human carcinogen by the International Agency for Research on Cancer in 1997 (IARC, 1997). The IARC designation of crystalline silica as a carcinogen was supported by epidemiologic evidence in man and pulmonary carcinogenicity in experimentally exposed rats (Johnson et al., 1987; Spiethoff et al., 1992; Muhle et al., 1995; Smith et al., 1995; Driscoll et al., 1997; IARC, 1997; Nehls et al., 1997; Steenland & Stayner, 1997; Tsuda et al., 1997; Rice et al., 2001; Steenland et al., 2001; Steenland & Sanderson, 2001; Attfield & Costello, 2004). The mechanisms underlying silica exposure effects on metabolism of PAHs remain unknown. In the absence of PAHs, silica alone increases CYP1A1, while decreasing the major constitutive CYP of the rat lung, CYP2B1 (Miles et al., 1993, 1994). However, the effect of silica alone on CYP1A1 and CYP2B1 activity was seen only when the silica was washed with HCl to remove surface contaminants (Miles et al., 1994). Another concern is that in the absence of the PAH substrate, perturbations in CYP1A1 activity do not alter PAH metabolites because there is no PAH available to be metabolized. Silica exposure causes both hypertrophy and hyperplasia of alveolar type II cells in the lungs of

exposed rats (Miller et al., 1987; Kawada et al., 1989; Miller & Hook, 1990). Previous microdissection studies have demonstrated that the highest levels of pulmonary CYP1A1 and CYP2B are in the parenchymal region of the lung, where alveolar type II cells are located (Lee et al., 1998). In rats treated with the model PAH β-naphthoflavone (NF), cell isolation experiments have demonstrated high CYP1A1 activity in the alveolar type II enriched populations (Lacy et al., 1992). Thus, if the hyperplastic and hypertrophied alveolar type II cells of silicosis had highly inducible CYP1A1 or CYP2B1, the silicotic lung could have a greater capacity for CYP1A1 induction than the normal lung. This resulted in the hypothesis that the hypertrophied and hyperplastic alveolar type II cells of silicosis are a site of CYP1A1 induction in the lung.

To investigate the influence of silica exposure in mixed exposures that include PAH, it was of interest to determine whether silica modifies CYP1A1- and CYP2B1-dependent xenobiotic metabolism in the lung. For this initial study of mixed exposure, silica exposures were used that were comparable to exposures causing acute and accelerated silicosis in humans, diseases characterized by alveolar epithelial cell hypertrophy and hyperplasia (Castranova & Vallyathan, 2000). Specifically, an autopsy specimen from a worker with accelerated silicosis had a silica lung burden of 21 mg/g dry lung, approximately 6 g/lung, a measured lung burden that was slightly higher than the 4 g/lung predicted by his occupational exposures (Seaton & Cherrie, 1998). The exposures used in this study were scaled down to the approximately 1000-fold smaller lungs of the rat, with the lowest exposure at 5 mg/lung, comparable to the lung burden in the human case of accelerated silicosis. Acute silicosis in humans is believed to involve higher silica exposures than accelerated silicosis, resulting in the exposure being set fourfold higher, at 20 mg/lung, for the initial experiments. This exposure was also comparable to the exposures previously used to investigate the effects of silica alone on CYP activity in the rat lung (Miles et al., 1993, 1994). This study focused on the effect of such high silica exposures upon PAH-dependent induction of pulmonary CYP1A1 protein and activity.

MATERIALS AND METHODS

Preparation of Silica

Crystalline silica (Min-U-Sil, U.S. Silica, Berkeley Springs, WV) was fractionated to <5 μm using a Donaldson particle classifier. By x-ray diffraction (XRD) it was 99.8% crystalline silica and by x-ray spectrometry (particle analysis) it was 98.7% crystalline silica. The mean surface area by nitrogen absorption was 4.7 m^2/g. The mass median aerodynamic diameter (MMAD) was 3.5 μm. The silica was sterilized by heating overnight at 160°C. The silica was suspended in 0.9% sodium chloride (Abbot Laboratories, North Chicago, IL) to produce 5, 10, or 20 mg/0.5 ml solutions for instillation.

Preparation of CYP Inducers

Stock solutions of 100 mg phenobarbital/ml (PB; Sigma Chemical, St. Louis, MO) and 50 mg β-naphthoflavone/ml (NF; Sigma Chemical, St. Louis, MO) were prepared in filter-sterilized corn oil and then sonicated for at least 20 min.

Animals

Male Sprague-Dawley Hla:(SD)CVF rats (Hilltop Lab Animals, Scottdale, PA), weighing between 125 and 150 g on arrival for these experiments, were used. Rats were housed in shoebox cages supplied with HEPA-filtered laminar flow air with Alpha-Dri Virgin cellulose chips (Shepherd Specialty Papers, Watertown, TN) and hardwood Beta-chips (NEPCO, Warrensburg, NY) for bedding, and were provided tap water and autoclaved ProLab 3500 (Purina Mills Incorporated, St. Louis, MO) rodent diet ad libitum. Rats were acclimatized in the AAALAC-accredited, barrier animal facility for 1 wk prior to use. The animals were treated humanely and with regard to alleviation of suffering. The experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee.

Exposure to Silica Followed by Model Inducers of CYP1A1 and CYP2B1

On d 1, rats were intratracheally instilled with 20 mg silica or saline (control). In the NF-exposed groups, 50 mg NF/kg was injected on d 12 and corn oil on d 14. In the PB-treated groups, PB (100 mg/kg) was injected on d 12 and d 14. In the combined NF/PB group, both NF and PB were injected on d 12, and PB on d 14. This resulted in 8 groups of rats: (1) control rats (n = 5); (2) rats with NF-induced pulmonary CYP1A1 (n = 5); (3) PB-exposed rats (n = 4); (4) combined NF/PB-exposed rats (n = 5); (5) silica-exposed rats (n = 4); (6) silica-exposed rats with CYP1A1 induced by NF (n = 3); (7) silica and PB-exposed rats (n = 4); and (8) silica, PB- and NF-exposed (n = 4). Rats were sacrificed on d 15; the right lung was used for microsomes, and the left lung was used for histopathology and immunohistochemistry.

Exposure to Silica and NF for Immunofluorescent Localization of Alveolar CYP1A1 Induction in Acute Silicosis

This experiment was designed to localize sites of CYP1A1 expression within the alveolus and compare changes at sites of CYP1A1 expression with the amount of CYP1A1 (Western blot) and the activity of CYP1A1 (7-ethoxyresorufin *O*-deethylase [EROD)]. CYP2B1 (PROD) activity was measured for comparison. On d 1, 12 rats were intratracheally instilled with 20 mg silica and another 12 rats with saline. On d 12, 6 rats were (ip) injected from each group with 50 mg NF/kg or vehicle alone (corn oil). Rats were sacrificed on d 15. Four experimental groups with n = 6 were formed: saline/corn oil-exposed

(control), saline/NF-exposed, silica/corn oil-exposed, and silica/NF-exposed. Right and left lungs were used for microsomes and pathology samples, respectively.

Silica Dose-Response Study in NF-Exposed Rats

This experiment was designed to determine the dose dependency of silica-associated downregulation of NF-induced CYP1A1 and CYP2B1 expression and activity. The lowest silica exposure was based upon the lung burden in accelerated silicosis, which was 4 or 6 g per lung in a human, for the calculated lung burden from exposure estimates and measured lung burden, respectively (Seaton & Cherrie, 1998), scaled down to the approximately 1000-fold smaller rat lung. To model the higher silica exposures believed to cause acute silicosis, the highest exposure was the 20-mg/rat exposure used in previous rat studies (Miles et al., 1993, 1994). Five rats were intratracheally instilled with saline, 5 mg silica, or 10 mg silica, and 4 rats with 20 mg silica. On d 12, each rat was injected ip with 50 mg/kg NF. Rats were sacrificed on d 15. Right and left lungs were used for microsomes and pathology samples, respectively.

Measurement of CYP1A1 and CYP2B1 Protein and Activities

Microsomes were prepared as previously described (Ghanem et al., 2004; Ma et al., 2002; Miles et al., 1993). Microsomal CYP1A1 and CYP2B1 protein were quantified by Western blots as previously described (Ghanem et al., 2006). Film images were scanned using the Eagle Eye II (Stratagene, La Jolla, CA) and quantified using commercial morphometry software (Image Quant 5.1 Molecular Dynamics/Amersham Biosciences, Piscataway, NJ). EROD (7-ethoxyresorufin *O*-deethylase), PROD (7-pentoxyresorufin *O*-deethylase), and ECOD (ethoxycoumarin *O*-deethylase) activities were measured as previously described (Ullrich & Weber,1972; Burke et al., 1985; Miles et al., 1993; Ma et al., 2002). In the experiment on the effects of silica and model inducers of CYP1A1 and CYP2B1, EROD activity was originally calculated as the total picomoles resorufin per milligram microsomal protein in the 8-min reaction period for direct comparability to the findings of Miles et al. (1993, 1994). This data was subsequently divided by 8 (total minutes in reaction period) to be comparable to current findings from multiple laboratories. In the remaining experiments, EROD activity was expressed as a rate (pmol resorufin/mg microsomal protein/min) as previously described (Ghanem et al., 2004, 2006). The bicinchonicic acid method (Pierce, Rockford, IL) was used to measure microsomal protein (Ma et al., 2002; Smith et al., 1985).

Histopathology

Left lung sections were processed and embedded in paraffin within 24 h. Five-micrometer sections were stained with hematoxylin and eosin and Masson's trichrome (Masson, 1929). Additional sections were placed on silane-treated slides (ProbeOn Plus, Fisher Scientific, Pittsburgh, PA) for immunostaining.

To evaluate silicosis, lung sections were examined by a board-certified veterinary pathologist (A. F. Hubbs) blinded to exposure status. Lungs from the dose-response experiment were scored semiquantitatively for severity and distribution of morphologic change as previously described (Hubbs et al., 1997). Briefly, the severity of morphologic injury was scored on a scale of 0 to 5 (none = 0, minimal = 1, mild = 2, moderate = 3, marked = 4, and severe = 5). The distribution (extent) or morphologic injury was also evaluated using a scale of 0 to 5 (none = 0, focal = 1, locally extensive = 2, multifocal = 3, multifocal and coalescent = 4, and diffuse = 5). The pathology score was the sum of the severity and distribution scores.

Enzymatic Immunohistochemistry

Enzymatic immunohistochemistry was performed using a minor modification to the manufacturer's streptavidin–biotin procedure (LSAB2 Kit, DAKO, Carpinteria, CA). The primary antibody, rabbit anti-rat CYP1A1/1A2 (Amersham, Buckinghamshire, England), was reconstituted using 300 μl of 1% bovine serum albumin (BSA) in phophate-buffered saline (BSA/PBS) and diluted 1:4 with antibody diluent (DAKO). Nonspecific staining was blocked with 3% hydrogen peroxide and tissue conditioner (Biomeda, Foster City, CA) and incubated overnight at room temperature with the primary antibody or rabbit serum (negative control). The secondary antibody was biotinylated anti-rabbit immunoglobulin; streptavidin peroxidase was used for labeling; the chromogen was 3-amino-9-ethylcarbazole (DAKO). The counterstain was aqueous hematoxylin (Biomeda, Foster City, CA).

The same procedure was used for CYP2B1 as described already for CYP1A1 immunohistochemistry except for minor differences in antibody preparation. The primary antibody, rabbit anti-rat CYP2B1, (Amersham) was reconstituted with 300 μl of 1% BSA/PBS, diluted 1:10 with antibody diluent (DAKO) and dropped on the slides.

Quantifying Airway and Alveolar CYP1A1 and CYP2B1 Expression

The number of CYP1A1- or CYP2B1-expressing nonciliated airway epithelial cells per millimeter basement membrane were counted in a minimum of 8 airways per lung using commercial morphometry software (Image-1, Universal Imaging, West Chester, PA). Ten different frames of alveolar regions measuring 0.035 mm^2 each were examined, and the CYP1A1- or CYP2B1-expressing cuboidal epithelial cells consistent with alveolar type II cells were counted.

CYP1A1/Cytokeratin 8 Immunofluorescence

In the alveolus, cytokeratin 8 principally localizes to alveolar type II cells (Kasper et al., 1993). In contrast to surfactant markers, cytokeratin 8 does not appear in macrophages during alveolar lipoproteinosis, which commonly occurs in silicosis (Hook, 1991). To localize alveolar CYP1A1 expression relative to type II cells, dual immunofluorescence was performed for CYP1A1 and cytokeratin 8, as previously described (Ghanem et al., 2004) with minor modifications. Polyclonal rabbit anti-rat CYP1A1 (Xenotech, Kansas City, KS) was used for staining CYP1A1 and polyclonal sheep anti-human cytokeratin 8 (The Binding Site, Birmingham, UK) for cytokeratin 8. Cytokeratin 8 was diluted 1:10. The other primary antibody, CYP1A1, was diluted 1:5 with the 1:10 diluted cytokeratin 8 to produce a suspension containing 1:5 CYP1A1 and 1:10 cytokeratin 8. The secondary antibodies, Alexa 594 goat anti-rabbit immunoglobulin (IgG) super highly cross-absorbed and Alexa 488 donkey anti-sheep IgG (Molecular Probes, Eugene, OR), were diluted 1:20 with PBS. Using this procedure, CYP1A1 fluoresced red and cytokeratin 8 fluoresced green.

Quantifying Alveolar CYP1A1 and Cytokeratin 8 Immunofluorescence

CYP1A1 and cytokeratin 8 staining was quantified in alveoli using digital images and commercial software (Metamorph, Universal Imaging, West Chester, PA) as previously described (Ghanem et al., 2004). Alveoli were classified as being in either the proximal alveolar region (microscopic field visibly adjacent to terminal bronchioles) or the random alveolar regions (random samples of alveolar fields not visibly near a terminal bronchiole). An average of five images each was calculated for proximal alveolar regions and random alveolar regions for each rat. Random alveolar samples have a >90% probability of being more than 3 alveolar duct generations from the bronchoalveolar junction (Mercer & Crapo, 1993).

The following measurements were made:

1. Percent region expressing CYP1A1.
2. Area within the alveolus expressing CYP1A1.
3. Percent region containing cytokeratin 8.
4. Area within the alveolus that contained cytokeratin 8.
5. Area of the region that coexpressed cytokeratins 8 and CYP1A1 (alveolar type II cells expressing CYP1A1).

The proportional expression of CYP1A1 was calculated in alveolar type II cells. This is the alveolar area with CYP1A1 colocalized with the type II cell marker, cytokeratin 8, divided by the alveolar area expressing cytokeratin 8. This calculation normalizes CYP1A1 expression in type II cells for the increase in type II cell size and number that is characteristic of silicosis (Miller & Hook, 1990).

Statistical Analysis

SAS Version 8.2 was used for all statistical analyses. Data from the first experiment (using both potential CYP-inducers) were analyzed as a three-way analysis of variance (ANOVA) comparing exposure to silica, CYP1A1 inducer, and CYP2B1 inducer. Pairwise comparisons were done using Fisher's least significant difference (LSD) test. The second experiment (localizing CYP1A1 expression) was analyzed using a two-way ANOVA comparing silica exposure and CYP1A1 inducer effects. Fisher's LSD was used for pairwise comparisons. A one-way ANOVA was used to compare the effect of silica exposures on CYP1A1 and CYP2B1 protein expression and activity in NF-exposed rats in the dose-response experiment. Histopathology scores were analyzed nonparametrically by the Kruskal–Wallis test. Values of $p < .05$ were defined as statistically different.

RESULTS

Histopathology

Silicosis was observed in silica-instilled rats but not in saline controls 2 wk after instillation (Figure 1). Morphologic changes associated with silicosis were significant alveolitis, lipoproteinosis, type II cell hypertrophy and hyperplasia, and granuloma formation. A dose-response relationship was not observed among the silica-exposed groups (Figure 2).

Exposure to Silica (Intratracheally) Followed by Model Inducers of CYP1A1 and CYP2B1: Immunohistochemistry

The number of CYP2B1-expressing cuboidal cells in alveoli and airways was unaffected by silica, NF, PB, or combinations of these exposures. However, the silica-exposed lungs contained more cuboidal alveolar cells than control

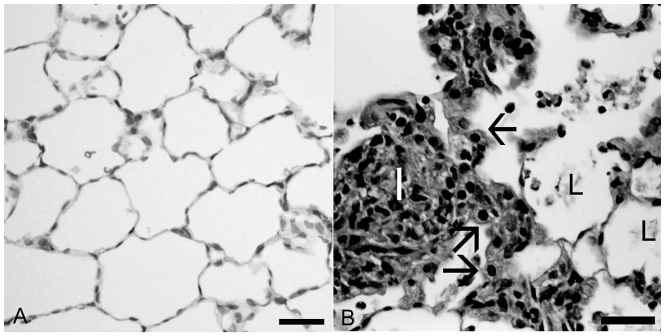

FIG. 1. Pulmonary histopathology from control and silica and NF-exposed rat lungs at 2 wk after silica exposure. (A) Alveolar region of the lung of a vehicle control rat. (B) Alveolar region of a rat exposed to silica and NF showing classic silica-induced changes. Morphologic changes include lipoproteinosis (L), type II cell hyperplasia (arrows), and inflammation (I) (H&E, bar = 30 μm).

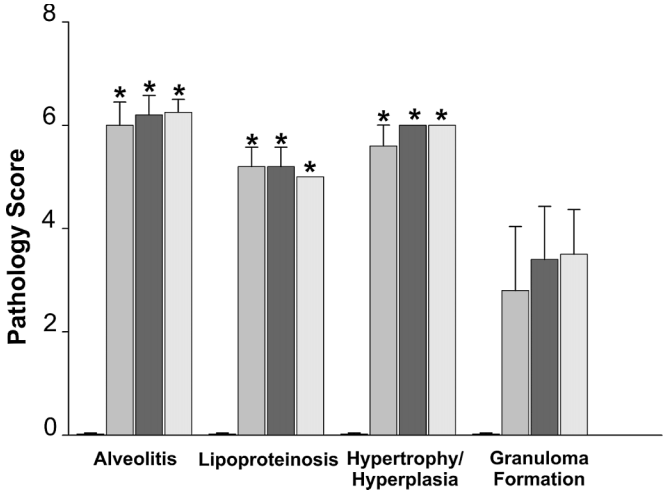

FIG. 2. Pathology scores for the morphologic changes in lungs of rats treated with 50 mg NF/kg and varying doses of silica. Values shown are mean ± SE. □ saline, ▒ 5 mg silica, ■10 mg silica, ▒ 20 mg silica. Asterisk denotes significantly different from control ($p \leq .05$).

lungs. Many of these cuboidal cells were larger than alveolar type II cells seen in control lungs and were morphologically consistent with the hyperplastic and hypertrophied alveolar type II cells of silicosis (Miller et al., 1987; Kawada et al., 1989; Miller & Hook, 1990). These hypertrophied cells consistently remained devoid of immunohistochemically detectable CYP2B1 (Figure 3A). In addition to expression of CYP2B1 in cuboidal cells, the alveolar septum between these measurable cuboidal cells also often expressed CYP2B1, but due to the indistinct margins of these cells, this expression was difficult to quantify on an individual cell basis.

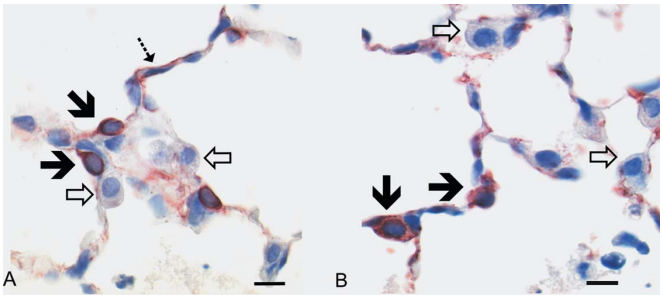

FIG. 3. Immunohistochemistry of pulmonary alveoli in rats exposed to silica and NF. (A) CYP2B1 immunohistochemistry. Cuboidal alveolar epithelial cells expressing CYP2B1 (black arrows) were found in all groups. CYP2B1 also appeared to be expressed in the alveolar septum that did not contain type II cells (dotted arrow). Only silica-exposed rats had cuboidal alveolar cells without CYP2B1 (open arrows; bar = 10 μm). (B) CYP1A1 immunohistochemistry. Alveolar epithelial cells expressing CYP1A1 (black arrows) were only found only in NF-exposed groups. Cuboidal alveolar cells without CYP1A1 expression (open arrows) were found in NF-treated rats exposed to silica (bar = 10 μm).

CYP1A1 expression was consistently observed in alveoli and airways of rats receiving NF. Without NF exposure, no immunohistochemical evidence of CYP1A1 was observed. In airways, NF exposure produced CYP1A1 expression in nonciliated airway epithelial cells. In the vasculature, NF exposure induced CYP1A1 expression in endothelial cells. In the alveolus, the thin cytoplasm of alveolar type I cells appeared to contain immunoreactive CYP1A1, although reactivity of alveolar capillary endothelial cells could not be excluded. This suggested the need for immunofluorescence and area measurements in follow-up experiments so that the contribution of these cells to lung CYP1A1 expression could be measured in future experiments. A few alveolar lining cells with the morphologic appearance of alveolar type II cells contained CYP1A1. However, the hypertrophied alveolar type II cells that are characteristic of silicosis did not express immunohistochemically detectable CYP1A1 (Figure 3B).

Morphometry confirmed that silicosis did not significantly affect the number of airway or cuboidal alveolar epithelial cells (presumptive alveolar type II cells) expressing CYP1A1 after NF exposure (Figure 4). Due to the thin cytoplasm of alveolar type 1 cells and alveolar capillary endothelial cells, the number of these cells expressing CYP1A1 could not be counted by morphometric analysis of immunohistochemically stained sections. PB, an inducer of hepatic CYP2B1, had no effect on pulmonary CYP1A1 expression.

Exposure to Silica (Intratracheally) Followed by Model Inducers of CYP1A1 and CYP2B1: CYP-Dependent Enzymatic Activity

Both ECOD (CYP-dependent) and EROD (CYP1A1-dependent) activities were significantly increased by NF exposure. However, induction of EROD and ECOD activity by

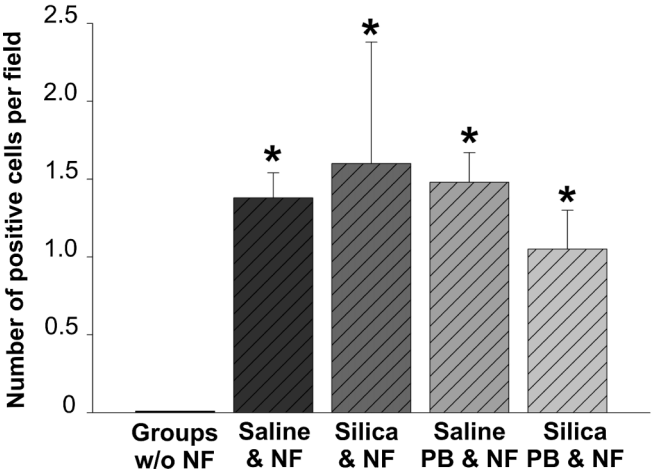

FIG. 4. Morphometry of CYP1A1 immunohistochemistry. The number of CYP1A1 expressing cuboidal alveolar cells (presumptive type II cells) was increased after NF exposure but was not significantly increased by phenobarbital or silica. Values shown are mean ± SE. Asterisk denotes significantly different from all groups without NF ($p \leq .05$).

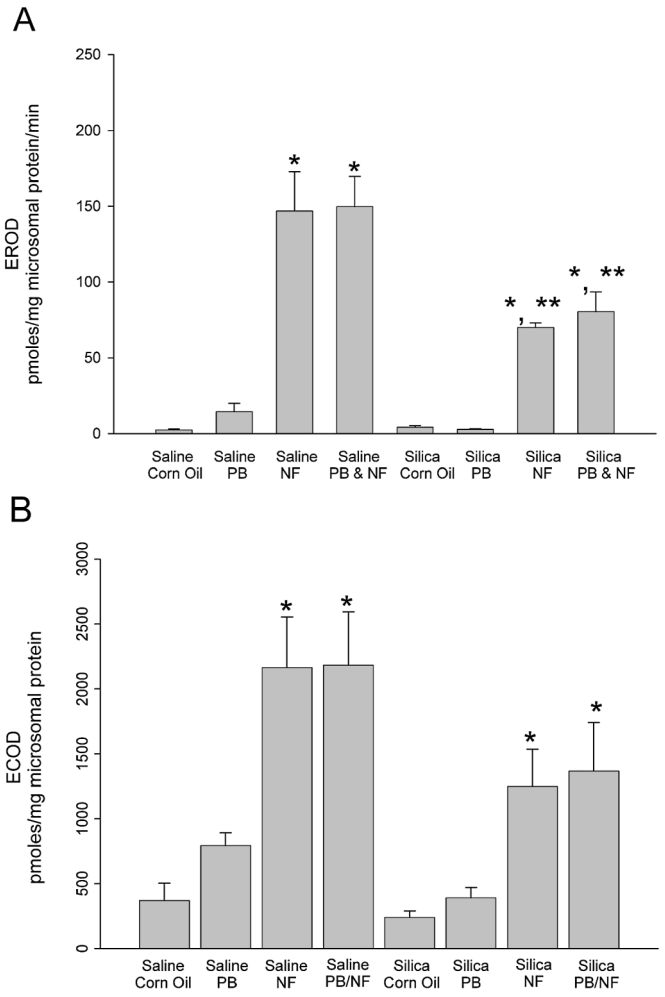

FIG. 5. Lung EROD and ECOD activity in vehicle controls and after treatment with silica, PB, and/or NF. (A) NF increased EROD activity. Silica exposure significantly decreased the magnitude of EROD induction. (B) ECOD increased in NF-exposed rats. Acute silicosis significantly attenuated NF-induced ECOD activity. Values shown are mean ± SE. Asterisk denotes significantly different from all groups without NF ($p \leq .05$); double asteriks, significant decrease from saline/NF and saline/NF/PB groups ($p \leq .05$).

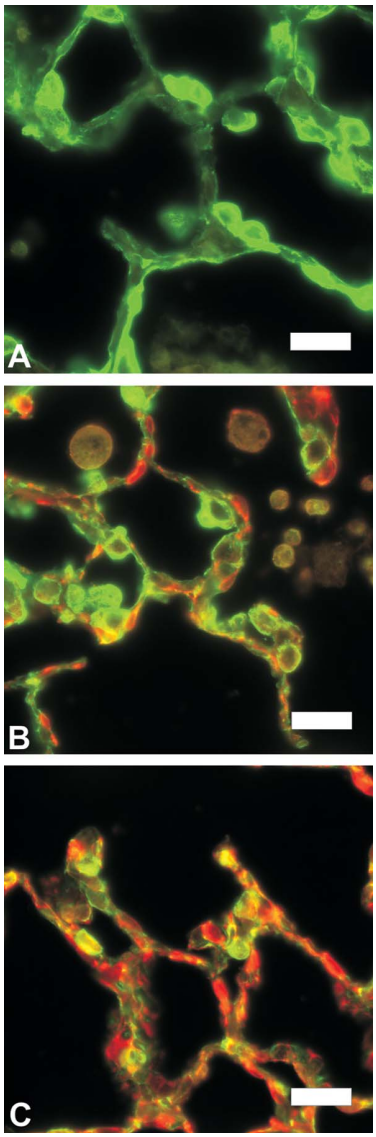

FIG. 6. CYP1A1 and cytokeratin 8 immunofluorescence in proximal alveolar regions of silica and NF-exposed rats. (A) In silica/corn oil-exposed rats, alveolar type II cells express cytokeratin 8 (green) but CYP1A1 (red) is not expressed (bar = 20 μm). (B) In silica/NF-exposed animals, abundant alveolar type II cells are indicated by cytokeratin 8 expression (green), and CYP1A1 expression is indicated in red. Yellow cells express both cytokeratin 8 and CYP1A1 (bar = 20 μm). (C) In the alveolar septum of saline/NF rats, more cells express CYP1A1 than in silica/NF rats (bar = 20 μm).

NF was significantly inhibited by prior intratracheal administration of silica (Figure 5, A and B).

Immunofluorescent Localization of Alveolar CYP1A1 Induction in Acute Silicosis

In rats exposed to silica followed by NF, immunofluorescence localized the site of alveolar CYP1A1 expression as well as the sites of cytokeratin 8 expression. Since cytokeratin 8 is expressed in alveolar type II cells, where CYP1A1 expression was colocalized with cytokeratin 8, the CYP1A1 expression was in alveolar type II cells. Both control and silica-exposed rats had cuboidal cells that were morphologically consistent with alveolar type II cells and fluoresced green, indicating cytokeratin 8 expression (Figure 6, A–C). Pulmonary CYP1A1 was not observed in the absence of NF (Figure 6A), while intense red fluorescence consistent with induction of CYP1A1 was seen in all rats receiving IP NF (Figure 6, B and C). Most of the CYP1A1 expression was within the alveolar septum, in cells that were not alveolar type II cells and were not alveolar macrophages. As noted in the immunohistochemistry study, these cells were morphologically consistent with alveolar type I or endothelial cells.

In the *proximal alveolar region*, cytokeratin 8 expression was significantly increased in groups exposed to silica (Figure 6, A and B, and Figure 7A). In NF-exposed rats, the amount of CYP1A1 expressed in type II cells was not significantly changed by silica exposure. However, there were more type II cells in silica-exposed rats, and the proportional expression of CYP1A1 in type II cells (the amount of CYP1A1 expressed in type II cells corrected for increases in type II cell number) was significantly reduced in rats exposed to both NF and silica compared to NF-exposed rats without silicosis (Figure 7B). The most striking observation was the appearance of many type II cells without CYP1A1 expression (Figure 6B).

In *random alveolar regions*, cytokeratin 8 was expressed in all groups but did not significantly differ between groups (Figure 8, A–C, and Figure 9A). Silica did not alter the area of CYP1A1 expression in random alveolar areas (Figure 9B).

The Effect of Silicosis on CYP1A1 and CYP2B1-Dependent Activity in NF-Exposed Rats

CYP1A1-dependent EROD activity significantly increased in groups that received NF. EROD activity of silica/NF groups

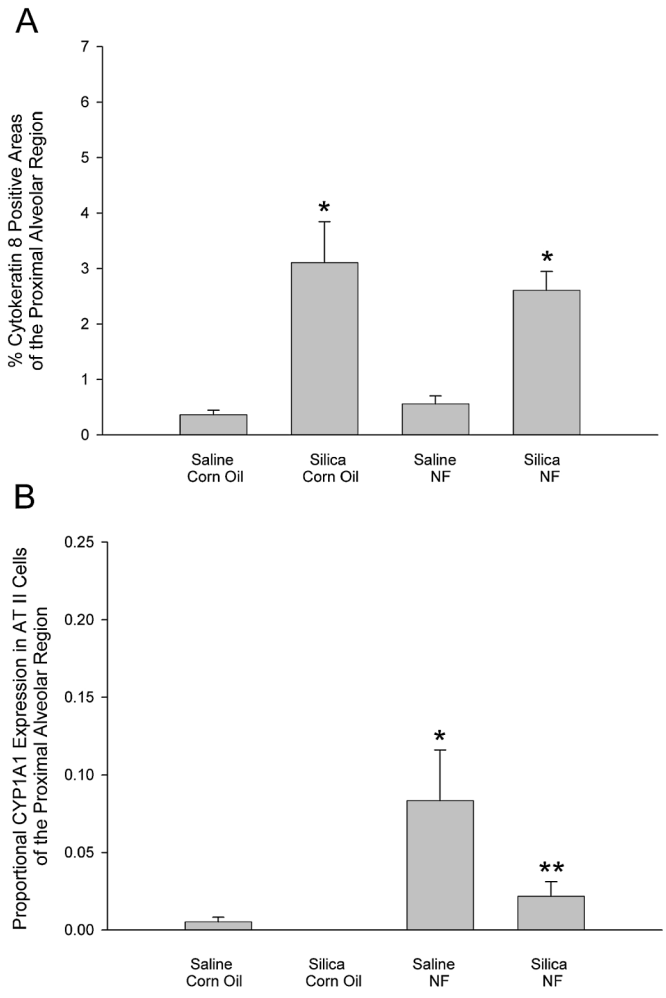

FIG. 7. Morphometry of CYP1A1 and cytokeratin 8 immunofluorescence in proximal alveolar regions. (A) Silica increased the alveolar area containing cytokeratin 8-expressing cells in the proximal alveolar region. (B) By morphometric evaluation, silica exposure decreased the proportional CYP1A1 expression in type II cells of NF-treated rats. Values shown are mean ± SE. Asterisk denotes significantly different from vehicle control (saline/corn oil) group ($p \leq .05$); double asterisk, significantly different from rats without silica exposure in the same NF-exposure group ($p \leq .05$).

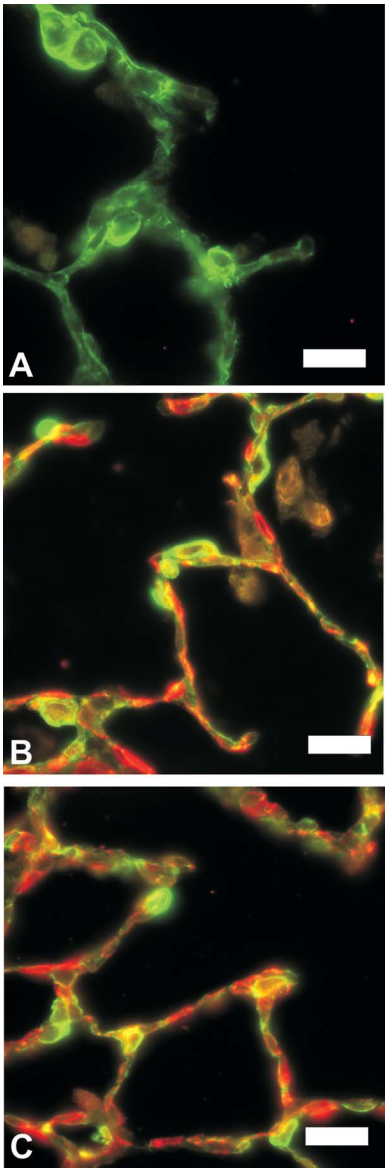

FIG. 8. CYP1A1 and cytokeratin 8 immunofluorescence in random alveolar regions. (A) Vehicle control rats did not express CYP1A1. Intense fluorescent green staining indicated cytokeratin 8 expression in type II cells (bar = 20 µm). (B) Random alveoli of rats exposed to NF and silica had both CYP1A1 expression (red) and alveolar type II cells (green) (bar = 20 µm). (C) Random alveoli of rats treated with NF alone also had both CYP1A1 expression (red) and alveolar type II cells (green). Bar = 20 µm.

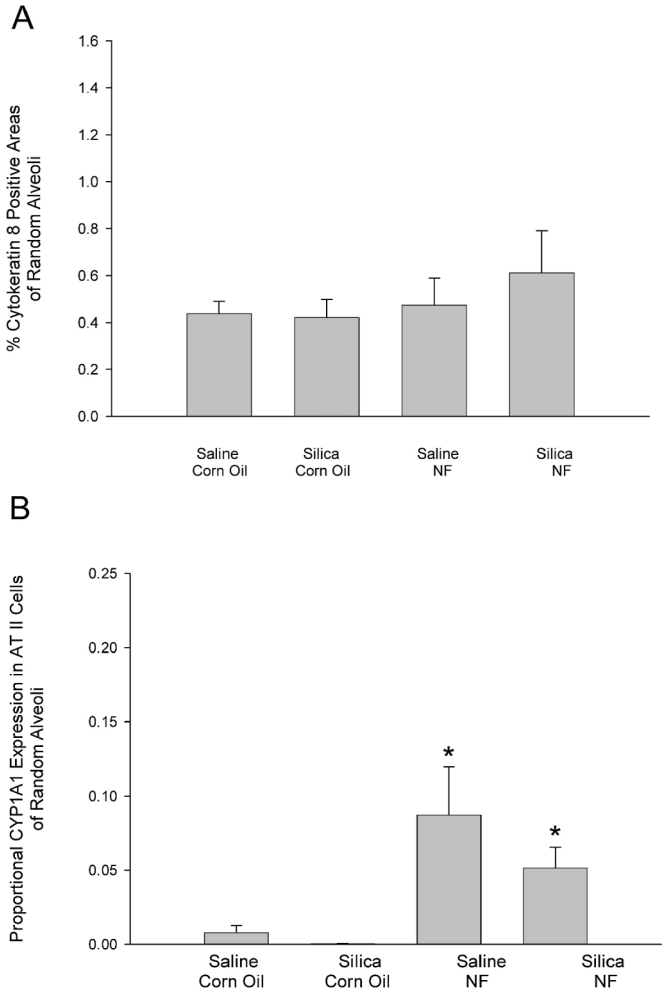

FIG. 9. Morphometry of CYP1A1 and cytokeratin 8 immunofluorescence in random alveolar regions. (A) By morphometric evaluation, cytokeratin 8 expression in random alveoli is unaffected by silica or NF exposure. (B) Proportional CYP1A1 expression in type II cells of random alveoli is increased by NF but unaffected by silica. The inhibition of CYP1A1 expression seen in proximal alveolar regions was not seen in random alveolar regions. Values shown are mean ± SE. Asterisk denotes significantly different from groups without NF ($p \leq .05$).

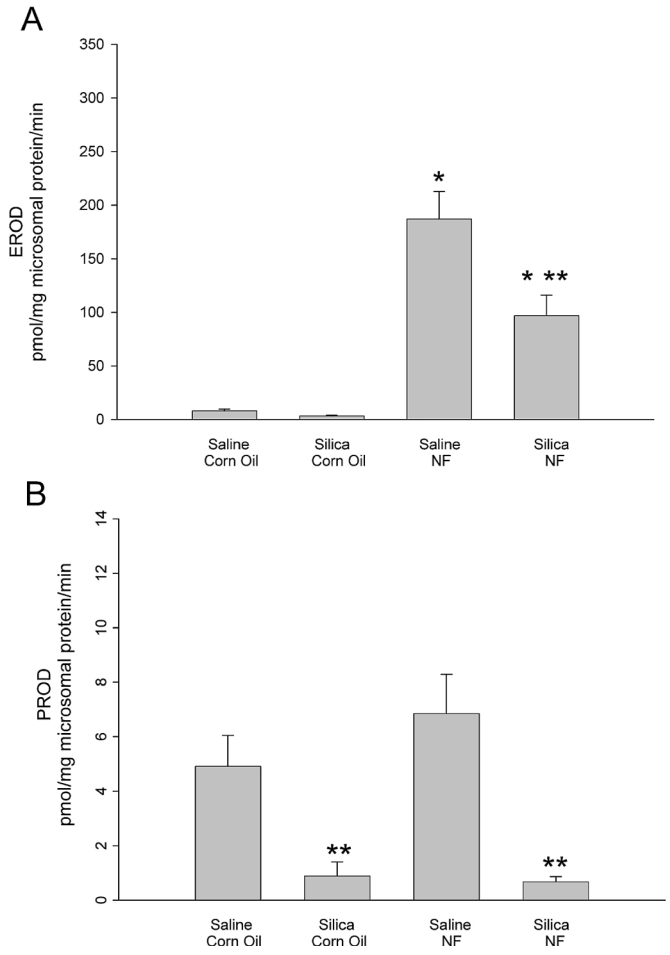

FIG. 10. The effect of silica on lung CYP1A1 and CYP2B1 protein and activity in NF-treated rats. (A) CYP1A1-dependent EROD activity is significantly induced by NF but silica significantly decreases NF-induced EROD activity. (B) Silica decreased CYP2B1-dependent PROD activity. Values shown are mean ± SE. Asterik denotes significantly different from groups without NF ($p \leq .05$); double asterisk, significantly different from rats without silica exposure in the same NF-exposure group ($p \leq .05$).

was significantly decreased compared to saline/NF groups (Figure 10A).

PROD (CYP2B1-dependent) activity was also significantly lower in groups exposed to silica when compared to saline groups (Figure 10B). NF did not affect PROD activity.

Silica Dose-Response Study with Exposure to Model Inducer of CYP1A1

Silica exposure significantly decreased CYP1A1-dependent EROD activity in NF-exposed rats (Figure 11A). This effect was fully manifested at the lowest exposure investigated (5 mg) without significant differences between silica-exposed groups. CYP2B1-dependent PROD activity was also significantly decreased in all silica-exposed groups. As with EROD, the suppression of PROD activity was fully manifested in rats exposed to 5 mg silica (Figure 11B).

The Effect of Silica Dose on CYP1A1 and CYP2B1 Proteins

In Western blots, silica significantly reduced CYP1A1 protein in NF-exposed rats. Western blots for CYP2B1 indicated that silica also significantly reduced CYP2B1 protein in NF-exposed rats. There were no significant differences amongst the silica-exposed groups for either CYP1A1 or CYP2B1 protein (Figure 12).

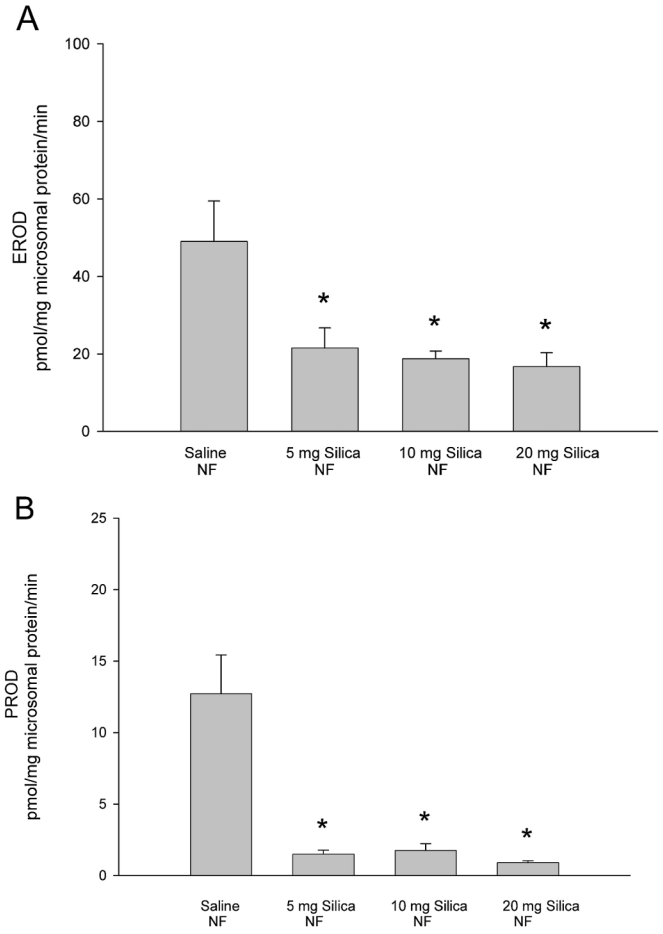

FIG. 11. Silica dose response of lung EROD and PROD activity. (A) Varying doses of silica have similar effects on EROD induction in NF-exposed rats. (B) Varying doses of silica have similar effects on PROD in NF-exposed rats. Values shown are mean ± SE. Asterisk denotes significantly different from rats exposed to NF alone ($p \leq .05$).

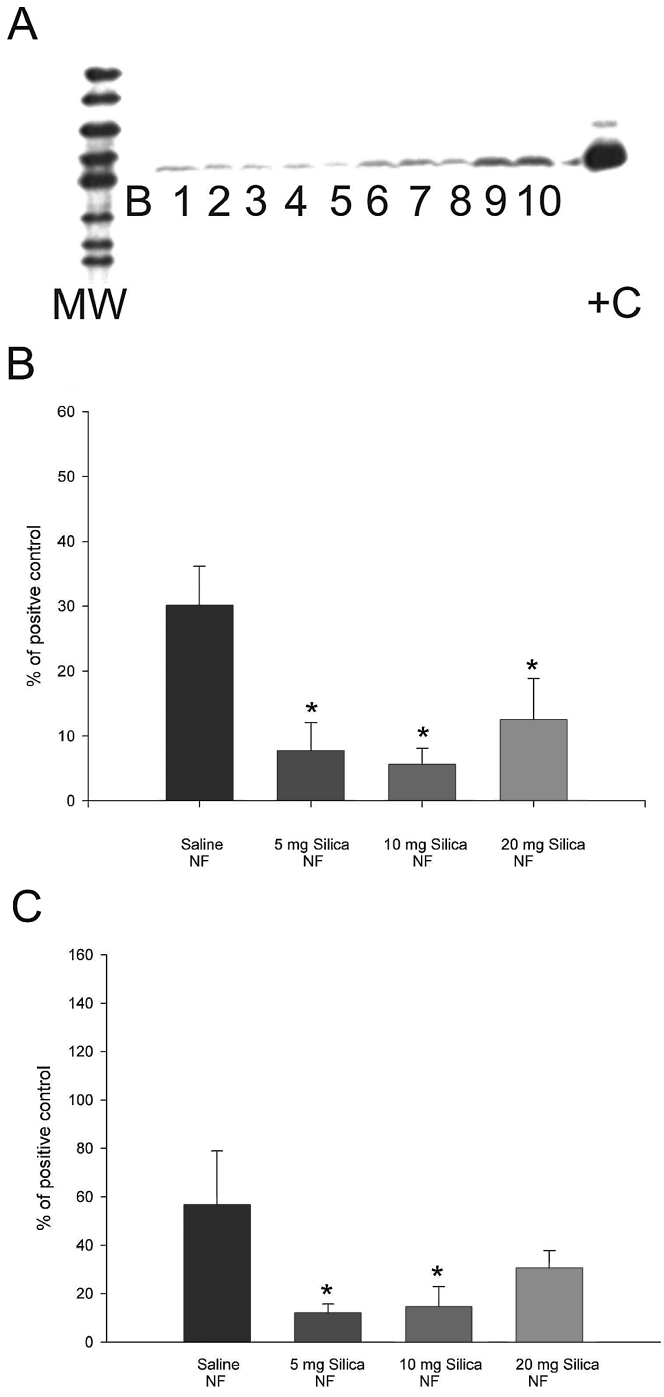

FIG. 12. CYP1A1 and CYP2B1 protein in Western blots of the NF- and silica-exposed lung. (A) Western blot for CYP1A1. Lanes 1 and 2: NF and 20 mg silica. Lanes 3, 4, and 5: NF and 10 mg silica. Lanes 6, 7, and 8: NF and 5 mg silica. Lanes 9 and 10: NF alone. (B) Graph quantifying densitometry scans for CYP1A1 from lung microsomes of rats exposed to NF alone or NF and varying amounts of silica. (C) Graph quantifying densitometry scans for CYP2B1 in lung microsomes of rats exposed to NF alone or NF and varying amounts of silica. MW, molecular weight marker; +C, positive control; B, blank. Asterisk denotes significantly different from rats exposed to NF alone ($p \leq .05$).

DISCUSSION

Our study provides the first insights into the influence of silica on CYP1A1 induction by a normal substrate, a PAH, NF. In this study, acute silicosis inhibited induction of CYP1A1 activity by NF and decreased CYP2B1 activity. In contrast, in previous studies, using a single-agent exposure, silica increased constituitive CYP1A1 expression (Miles et al., 1993, 1994; Becker et al., 2006).

In the previous studies, significant increases in CYP1A1 expression were observed after exposures to acid-washed, but not unwashed, silica in the absence of a PAH (Miles et al., 1993, 1994). In addition, silica increased CYP1A1 messenger RNA in the absence of PAHs in an immortalized rat epithelial cell line in vitro but not in lung epithelial cells isolated from in vivo exposed rats up to 90 d after silica exposure (Becker et al., 2006). However, at 180 to 360 d after exposure, increased

numbers of CYP1A1-expressing cells and some cells with nuclear aromatic hydrocarbon receptor (AhR) were observed by immunohistochemistry in silica-exposed rats (Becker et al., 2006). Thus, studies on the effect of silica alone in the absence of a substrate/inducer suggest that chronic silica exposure may increase substrate/inducer metabolism upon a subsequent single substrate/inducer exposure.

In our current study, we did not see a repeatable effect of silica alone upon CYP1A1 or CYP2B1 activity. This is consistent with the previous finding that silica-induced changes in CYP1A1 and CYP2B1 activity are only seen when acid-washed silica is studied (Miles et al., 1994). In preliminary studies in our laboratory using rats exposed to saline, acid-washed silica, or unwashed silica, the number of alveolar type II cells containing CYP2B1 and the extent of histopathologically assessed alveolar epithelial hyperplasia did not differ among the groups (Levy, 1995). Because there were no differences in those endpoints, and for consistency with most existing silica studies, unwashed silica was selected for studying the effects of mixed exposures to silica and PAHs. Importantly, the magnitude of the PAH-associated increased CYP1A1 activity was more than 10-fold greater than the total CYP1A1 activity in lungs of vehicle control rats or rats exposed to silica as a single agent. The PAH-associated induction of CYP1A1 was inhibited by silica in the mixed exposures used in the current study. In this mixed exposure to silica followed by NF, CYP2B1 activity was also reduced by silica.

The intraperitoneal route was used for NF exposure and CYP1A1 induction in this study. By this route, the absorbed NF is widely distributed via the vasculature, where it clearly reaches the lung, since CYP1A1 is induced there (Lindeskog et al., 1990). By inhalation, the most likely route of exposure in the workplace, the initial site of alveolar exposure is on the alveolar epithelium. However, half of the model PAH, benzo[a]pyrene, is cleared from the alveolar region into the blood in 2.4 and 4.3 min for crystalline and particle-adsorbed benzo[a]pyrene, respectively (Gerde et al., 1993; Gerde et al., 2001). PAHs clearly penetrate the alveolar septum by diffusion. Thus, the NF-induced CYP1A1 in this study appears to be a reasonable model for the PAH-exposed lung.

Workers with silicosis display increased lung inflammation and cytokine production (Vanhee et al., 1995). The silica lung burdens (5 to 20 mg/lung) used in our rat study were designed to range from the lung burden of 5 mg/g, similar to the ~6 mg/g burden producing accelerated silicosis in a human (Seaton & Cherrie, 1998), to the exposure used in previous studies investigating the effects of silica exposure on pulmonary CYP1A1 (20 mg/lung), which modeled the higher silica exposures believed to cause acute silicosis (Miles et al., 1993, 1994). Thus, these exposures were designed to produce lung burdens comparable to those of diseased workers and rats with silicosis, conditions with active inflammation (Porter et al., 2001). As with workers, these lung burdens produced histopathologically documented inflammation in the lungs of rats in our study. Several cytokines involved in lung inflammation, such as tumor necrosis factor (TNF)-α and nuclear factor (NF) κB, interfere with CYP1A1 induction (Paton & Renton, 1998; Tian et al., 1999; Ke et al., 2001). In addition, another inflammatory mediator, nitric oxide, binds the catalytic heme moiety of CYPs, directly reducing their activity at the protein level (Stadler et al., 1994). Experimental silicosis increases nitric oxide, TNF-α, and NFκB (Castranova et al., 2002). Our study showed that silica exposures, at doses producing lung inflammation, inhibit PAH-dependent CYP1A1 induction in vivo. This silica-associated inhibition of PAH-dependent CYP1A1 induction was observed as a suppression of (1) CYP1A1 activity, (2) CYP1A1 protein on Western blot, (3) CYP1A immunohistochemistry, and (4) CYP1A1 immunofluorescence, when compared with PAH exposure alone. Thus, the effect of silica as a component of this mixed exposure was the opposite of the effect of a single-agent silica exposure (Becker et al., 2006; Miles et al., 1993, 1994).

The ability of silica to inhibit NF-induced CYP1A1 was similar to the ability of coal dust to inhibit NF-induced CYP1A1 activity (Ghanem et al., 2004, 2006). In this silica study, similar to the study of coal dust, inhibition of NF-induced CYP1A1-induced CYP1A1 induction was seen at silica exposures producing pulmonary inflammation. This ability of two separate particles to inhibit CYP1A1 induction in the lung suggests that this may be a general effect of particle-induced inflammation, or potentially, a general effect of pulmonary inflammation. These hypotheses are additionally supported by the effects of cytokines noted above, and the role of inflammation in regulating CYP expression in the liver (reviewed in Morgan, 1997). The effects of low-dose mixed silica and PAH exposures were not investigated in this study, and, with the anticipated decreased inflammation at lower exposures, the effects of such exposures remain to be investigated. The suppression of CYP2B1 expression and activity observed was similar to that reported in the lungs of rats exposed silica alone or to other respirable particles (Miles et al., 1993; Ma & Ma, 2002).

Studies demonstrated that the proximal alveolar region was the site of specific cell changes. Alveolar type II cells in this region proliferated in response to silica exposure. Using immunofluorescence, proportional CYP1A1 expression within alveolar type II cells decreased in animals exposed to both silica and the CYP1A1 inducer NF when compared to animals exposed to just NF. This morphometric assessment corresponded with the immunohistochemical observation that the large hypertrophied type II cells of silicosis did not contain immunohistochemically detectable CYP1A1 or CYP2B1. These findings were also consistent with the observation that the site of particle deposition in intratracheally instilled rats is the proximal alveolar region (Brain et al., 1976). Thus, the site of particle deposition, the proximal alveolar region, was the site where CYP1A1 expression changed in alveolar type II cells. Similarly, the cells that did not express CYP2B1 in our immunohistochemistry experiment were morphologically consistent with the hypertrophied type II cells that replace alveolar type I cells damaged in silico-

sis (Miller & Hook, 1990). In contrast to our original hypothesis that the hypertrophied and hyperplastic alveolar type II cells of silicosis are a site of CYP1A1 induction in the lung, these cells were virtually devoid of inducible CYP1A1 expression. For this reason, in future investigations of the role of pulmonary inflammation on pulmonary CYP activity, inflammatory stimuli that do and do not cause alveolar epithelial cell hyperplasia are worthy of investigation. Consistent with the morphologic observation, CYP2B1- and induced CYP1A1-dependent activity was suppressed in silicotic rats.

The downregulation of CYP1A1 and CYP2B1 in acute silicosis may have important implications for cancer studies in humans exposed to both PAH and silica. CYP1A1 is an important enzyme involved in lung cancer initiation because it metabolizes PAHs to reactive intermediates. These reactive intermediates are generally more easily conjugated during phase II xenobiotic metabolism and their excretion is generally facilitated, but some products of CYP1A1-mediated PAH metabolism may react with DNA to produce DNA adducts and somatic mutations, with the potential initiation of cancer (Newbold & Brookes, 1976; Marshall et al., 1984). In cigarette smokers, who are exposed to PAHs, such as benzo[a]pyrene, in cigarette smoke, the risk of DNA adducts and lung cancer correlates with CYP1A1 activity in many studies (Kellermann et al., 1973; Bartsch et al., 1992, 1999; Mollerup et al., 1999, 2006). Similarly, CYP2B1 has been implicated in metabolizing promutagens into more mutagenic compounds in the rat lung (Zhao et al., 2004). Multiple studies also implicate silica as a pulmonary carcinogen (Smith et al., 1995; IARC, 1997; Steenland & Stayner, 1997; Tsuda et al., 1997; Rice et al., 2001; Steenland et al., 2001; Steenland & Sanderson, 2001; Attfield & Costello, 2004). The study presented here is important, because it suggests that silica may potentially downregulate CYPs implicated in activating carcinogens present in cigarette smoke, one of the major causes of human lung cancer. Our study suggests the possibility that silica may be a modifier, rather than a covariable, in PAH-induced lung cancer. This provides a potential theoretical basis for understanding some of the variability in the lung cancer epidemiology seen in silica exposed workers (Hessel et al., 2000). Additional studies will be needed to investigate whether CYP1A1 activity is also suppressed in the lungs of silica-exposed workers who are occupationally or avocationally exposed to PAHs.

In summary, this study indicates that acute silicosis inhibits (1) CYP2B1 and (2) the PAH-dependent induction of CYP1A1 expression and activity in the rat. In conjunction with previous studies showing that coal dust-associated downregulation of CYP1A1 induction is inversely proportional to the severity of pulmonary inflammation, these findings suggest that respirable dusts that produce pulmonary inflammation are potential modifiers of PAH-induced, CYP1A1-dependent, xenobiotic metabolism. Evidence indicates that respirable crystalline silica dust, like respirable coal dust, is a negative modifier of PAH-induced CYP1A1 activity in the rat lung.

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