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SUPPRESSION OF OVINE CYTOCHROME P4501A1 (CYP1A1) INDUCTION BY RESPIRABLE COAL DUST PARTICLES

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ABSTRACT

Respirable coal dust (CD) is a common cause of lung injury and pneumoconiosis in human and animals. Lung is an organ that is more susceptible to exposure to polycyclic aromatic hydrocarbons (PAHs), such as to tobacco products. The pulmonary toxicity and carcinogenicity of PAHs is dependent upon metabolic activation by cytochrome P4501A1 (CYP1A1), which is inducible by its own substrates and can activate the PAHs into reactive metabolites producing nucleotide adducts. Because preliminary results showed that the mixed exposure to both CD and PAHs reduced the metabolic activities of CYP1A1 in rats, we have examined the hypothesis that CD alters the localization and metabolic activity of CYP1A1 in the particle-exposed lung of ovine model. Therefore, the right apical lung lobes of lambs were instilled with 500 mg respirable CD (n=4) or saline (n=5) using a bronchoscope. The lambs were injected with 50 mg/kg of the model PAH, beta-naphthoflavone (BNF), on day 53 and 54 post-exposure and euthanized on day 56. CD exposure caused histiocytic inflammation extending from the terminal bronchioles into adjacent alveolar space and interstitium. In bronchoalveolar lavage (BAL) fluid of exposed lobes, 23 to 73% (mean 44.5%) of alveolar macrophages contained phagocytized CD. By immunofluorescence, the expression of CYP1A1 was significantly reduced within the alveolar septum. CYP1A1-dependent 7-ethoxyresorufin-O-dethylase (EROD) activity and CYP2B-dependent 7-pentoxyresorufin-O-deethylase (PROD) activity were both decreased in the pulmonary microsomes from CD-exposed lobes. These findings in a non-rodent model support the hypothesis that respirable CD modifies the induction and pulmonary localization of CYP1A1 protein.

INTRODUCTION

Preliminary data from experiments on rats suggest suppression of CYP1A1 induction and its dependent enzymatic activity (EROD) by pulmonary exposures to respirable CD (Ghanem et al, 2003). In addition, CD particles suppress the enzymatic activity (PROD) of the CYP2B1 isoform in rat lungs (Ghanem et al. 2003). However, the comparability of rat and human pulmonary responses to respirable particles of low toxicity has been questioned (ILSI, 2000). In this study, we used the lamb as a non-rodent model to establish the relationship between pneumoconiosis, caused by CD deposition in ovine lungs, and alterations in xenobiotic metabolism. Specifically, we have examined the activity, quantity, and localization of CYP1A1, the CYP isoform which activates some polycyclic aromatic hydrocarbons (PAHs), such as those in cigarette smoke, to produce DNA-binding carcinogenic intermediates (Bjelogrlic et al, 1993; Liang et al. 2003). In addition, we have studied the activity of CYP2B. CYP2B is the major constitutively expressed CYP member in sheep lung, which is analogous to CYP2B1 in rats (Williams et al. 1991). Lambs were selected as a model because the ovine response to respirable crystalline silica, another cause of pneumoconiosis, is similar to that observed in humans (Begin et al. 1989; Larivee et al. 1990). Ovine lungs are also large,

which permits directed exposure and the use of internal control lobes, an important advantages in an outbred species.

The sheep coding region of CYP1A1 has an 85% homology to human CYP1A1 (Hazinski et al. 1995). Therefore, sheep appear to be an appropriate model to study the effect of xenobiotics, such as PAHs, on CYP1A1-associated carcinogenic pathways. Our results showed that CD exposure reduced the potential for induction of CYP1A1-dependent (EROD) and CYP2B-dependent (PROD) enzymatic activities in lung microsomes of sheep exposed to a CD particle suspension and BNF, a CYP1A1 inducer. These results were further supported by Western blot analysis for CYP1A1 protein and immunofluorescence examination.

MATERIALS AND METHODS:

Sheep

Nine 3-month-old Katahdin crossbred castrated male lambs weighing 17-30 kg were used in this study. The lambs were housed in the Food Animal Research Facility (FARF) of West Virginia University. Lambs were fed Alfagreen Supreme Dehydrated alfalfa pellets (containing 17% crude protein, 1.5% crude fat and 30% crude fibers), with *ad libitum* supply of water. The lambs were kept for 3 weeks prior to exposure for acclimatization. During this period, they were examined and all parameters were within normal range. To assure parasite-free lambs, ivermectin was injected subcutaneously 3 weeks before instillation.

Experimental Design:

Lambs were randomized into a CD-exposed and a control group using a randomizing program (www.randomized.com). The lambs were exposed in groups of 2-3 lambs / day over an eight day period. The lambs were sacrificed eight weeks after exposure. All lambs were subcutaneously injected with 10 mg/kg Tilmicosin antibiotic (Mycotil, Eli Lilly, Indianapolis, IN) one day after instillation of the final lamb as a prophylaxis against pulmonary infections. Three and two days before sacrifice, lambs were intraperitoneally (IP) injected with 50 mg/kg BNF, to induce pulmonary CYP1A1.

Preparation of Particle Suspension

CD particles (< 5 micrometer in diameter, 500 mg/lamb) were heat sterilized for 2 h at 160 ºC. The particles used in this study contained 0.34 % total iron of which 0.119 % was surface iron. The CD suspension was prepared by addition of 15 ml sterilized saline to 500 mg CD and vortexed. The entire volume was used for intrapulmonary instillation. In control animals, 15 ml saline alone was instilled. The suspension was drawn into a sterile syringe attached to a 1 mm diameter polyethylene tube and inserted into an endoscope (Jorgensen Laboratories Inc., Loveland, CO). The whole amount was instilled in the right apical lobe. In the control group, only 15 ml of the sterile saline was instilled into the same lobe under the guidance of the fiberoptic bronchoscope.

Intratracheal Instillation of Particles Using Flexible Fiberoptic Bronchoscope

Lambs were anesthetized by intramuscular injection of ketamine hydrochloride (Keta-ject, Phoenix Laboratories Inc., St. Joseph, MO) 11 - 15 mg/kg and xylazine hydrochloride (Xyla-ject, Phoenix Laboratories Inc., St. Joseph, MO) 0.22 mg/kg.

A fiberoptic bronchoscope (Jorgensen Laboratories Inc., Loveland, CO) with a 5-mm external diameter was inserted though a speculum into the trachea and the CD suspension was instilled into the lumen of the bronchus of the right apical lobe. Importantly, in sheep, the right apical lobe is divided into 2 segments, the cranial segment and the caudal segment, each one receives a separate bronchus from the mainstem right tracheal bronchus (Getty, 1975). In our instillation procedure, the CD was instilled in the mainstem tracheal bronchus so that both segments were exposed. After instillation, lambs were kept on the right side to allow settling of particle suspension within the instilled lobe. To facilitate anesthetic recovery, yohimbine hydrochloride (Yobine, LLOYD Laboratories, Shenandoah, OH) was injected IV (0.2-0.4 mg/kg) after the lambs were placed in the recovery stall.

Lamb Necropsy

Lambs were euthanized by intravenous injection of Sodium Pentobarbital (Sleepaway®, Fort Dodge Animal Health, Fort Dodge, Iowa) 26 mg/lb. The entire lung was weighed and the right tracheal bronchial lobe and the left apical lobe were separately weighed. Both lobes were lavaged with PBS solution to collect the bronchoalveolar lavage fluid (BALF) as described later. Following lavage, 10 % of each lobe, by weight was excised for microsome preparation and the other 90 % was fixed by airway perfusion with a volume of 3 ml of 10 % neutral buffered formalin per gram lung.

Preparation of Lung Microsomes

Microsomes were obtained by differential centrifugation method as previously described (Flowers and Miles, 1991; Ma et al. 2002). These microsomes were used for measuring the CYP1A1 and CYP2B1-dependent enzymatic activities (EROD and PROD, respectively). In addition, lung microsomes were subjected to electrophoresis to determine the CYP1A1 protein by western blot analysis.

Determination of the Total Lung Proteins

The total amount of protein in the microsomes was determined by the bicinchoninic acid (BCA) method as previously described (Ma et al. 2002; Smith et al. 1985) using the BCA protein assay kit (Pierce, Rockford, IL) in a spectra Max 250 Spectrophotometer (Molecular Devices Corporation, Sunnyvale, California). Bovine serum albumin was used for the standard curve. Protein was measured as mg/ml.

Determination of CYP1A1- and CYP2B-Dependent Enzymatic Activities (EROD and PROD)

Spectrofluorometric assays for measuring EROD and PROD metabolic activities were performed as previously described (Burke et al. 1985; Ma et al. 2002) using a luminescence spectrometer model LS-50 (Perkin-Elmer, Norwalk, CT) and resorufin (Sigma, St. Louis, MO) as the standard. EROD and PROD activities were expressed as picomoles of the produced resorufin per minute per milligram microsomal protein (pmol/min/mg protein) as previously described (Ma et al. 2002).

Immunofluorescence Double Labeling

Fixed tissues were processed at the day of collection to preserve antigenicity. A representative slide from each lobe (the first section from the lobe) was used for immunofluorescent detection of CYP1A1 and the alveolar type II cell (AT-II) marker, cytokeratins 8/18. Slides containing 4-micrometer-thick tissue sections were heated in the oven at 60 ºC for 15 minutes. Deparaffinization and rehydration were routinely

conducted by sequential immersion in xylene and alcohol. Antigen availability was maximized by heating the slides in a microwave with 0.01M EDTA, pH 8.0. Non-specific binding was blocked by incubating the slides with 5% bovine serum albumen (Sigma- Aldrich Co.) (in PBS) for 10 minutes followed by incubation with 5% pig serum (Biomeda Corporation, Foster City, CA) for 10 minutes at room temperature. The primary antibodies were a polyclonal rabbit anti-rat CYP1A1 (Xenotech, Kansas City, KS) diluted 1:5 with PBS and a polyclonal Guinea pig anti-cytokeratins 8/18 (Researech Diagnostic Inc., Flanders, NJ) diluted 1:50 with PBS. Rabbit serum was applied as a negative control. After 48 h incubation at room temperature, the slides were incubated for additional 2 h at 37 ºC. The slides were then thoroughly washed to remove un-conjugated primary antibodies and the secondary antibodies were dropped onto the slides. The secondary antibodies mixture consisted of an equal volume of a FITC-labeled, donkey anti-Guinea pig IgG (Research Diagnostic Inc., Flanders, NJ) diluted 1:50 with PBS and an Alexa 594-conjugated goat anti-rabbit antibody (Molecular probes, Eugene, Oregon) diluted 1:20 with PBS. The slides were incubated with the secondary antibodies for two h in the dark at room temperature. The slides were visualized using a fluorescent photomicroscope (OlympusAX70, Olympus American Inc., Lake Success, NY) and images were captured using the 40x objective (an area of 34466.1 μm^2) and a Quantix cooled digital camera (Photometrics, Tucson, AZ) with QED camera plugin software (QED Imaging, Inc., Pittsburgh, PA). Five images were captured per slide from the proximal alveolar (PA) regions, where most of the CD particle tend to localize near the terminal bronchioles and alveolar ducts.

Morphometry

Immunofluorescence morphometric analysis using Metamorph software (MetaMorph, Universal Imaging Corp., Downingtown, PA) was conducted on digital images. The area of CYP1A1 expression was quantified in AT-II and alveolar septal cells that did not contain AT-II markers (NT-II) as follow:

1- Area of colocalization of CYP1A1 in AT-II

The area of CYP1A1 colocalized with cytokeratins 8/18 in AT-II was obtained by the following formula:

$$A = B \times C$$

Where: A = the area of CYP1A1 that co-expressed (colocalized) with cytokeratins 8/18 in AT-II,

B = the percent of CYP1A1 expressed in AT-II measured by the Metamorph software, and

C = the total CYP1A1 area expressed in the whole alveolar septum (including AT-II and non-AT-II) measured by the Metamorph software.

2- Proportional CYP1A1 expression in AT-II

The proportional CYP1A1 expression in AT-II, which is the relative area of CYP1A1 to cytokeratins 8/18 expression, was calculated from the following formula:

$$P = \frac{A \times B}{G}$$

Where: A and B are as defined above and,

G = the total green area of cytokeratins 8/18 expressed in AT-II cells.

P = the proportional CYP1A1 expression within areas occupied by the AT-II marker, and adjusts for increases associated with AT-II hyperplasia.

AT-II hyperplasia and hypertrophy were assessed by measuring the area of cytokeratins 8/18 expression.

3-Area of CYP1A1 expression NT-II

This area reflects the expression of CYP1A1 in NT-II and can be calculated from the following formula:

$$N = Q \times C$$

Where: N = the area of CYP1A1 expression in NT-II ,

Q = the percentage of CYP1A1 expression in NT-II measured by the Metamorph software,

C = as defined before.

Western Blot Analysis

The amount of CYP1A1 apoprotein in lung microsomes was determined by Western blot as previously described (Ma et al. 2002) with minor adaptation. A Novex Tris glycine gel with 15 small wells, (Invitrogen Corporation, Carlsbad, CA), and 30 µg of microsomal proteins were subjected to SDS gel electrophoresis for 90 min at 120 volts followed by transfer to a nitrocellulose membrane (blotting) for another 90 minutes at 25 volts. Liver microsomes of BNF-treated rat (Xenotech, Kansas city, KS) were used as a positive control. Non-specific binding was blocked by incubating the membrane with a 5% solution of dry milk in tris-buffered saline (TBS) for 1 h at room temperature with. The protein bands were identified by using a primary polyclonal rabbit anti- rat CYP1A1 antibody (Xenotech, Kansas city, KS) for overnight incubation at 4 ºC. The membranes were washed and incubated for 1 h with a HRP (horse radish peroxidase)-conjugated goat anti-rabbit IgG (Santa Cruz Biotech. Inc., Santa Cruz, CA) at room temperature. Super RX Fuji Medical X-ray film was then exposed to the membranes at room temperature. Band intensity was scanned by Eagle Eye II with Eagle Sight software (Strategene, La Jolla, California). The density was measured using ImageQuant software version 5.1 (Amersham Pharmacia Biotech, Piscataway, NJ). After quantification, the data were presented as a percentage of the positive control.

Bronchoalveolar Lavage Fluid (BALF) Analysis

Both the right apical (exposed) and left apical (internal control) lobes were lavaged using ice-cold PBS (Ca^{++} and Mg^{++} free). Three consecutive lung lavages were performed in which the first lavage was collected by using 2 ml/gm lung while the subsequent 2 lavages were conducted by using 2.7 ml/gm lung weight. The lobes were excised from the rest of lung and the incision site was clamped off by digital pressure during BAL collection process. Nevertheless, some of the lavage fluid was lost due to incision of the lung tissue which reduced the recovery of the fluid. Therefore, the BAL differential is considered more accurate than the total BAL cell yield. The BALF analysis was conducted as previously described (Porter et al. 1999).

BAL Cellular analysis

Alveolar macrophages (AM) and polymorphonuclear leucocytes (PMN) were counted by using a coulter multisizer II and AccuComp software (Coulter Electronics, Hialeah, Fl) as previously described (Castranova et al. 1990). Cytospin preparations of BAL cells of 1 x 10^5 total phagocytes (AM and PMN) suspended in 200 µl HEPES-

buffered solution were prepared by using a Shandon Elliot cytocentrifuge (800 rpm for 5 minutes). The cytospin preparations were stained with modified Wright-Giemsa stain as previously described, and cell differentials were determined by light microscopy (Porter at al, 20002a). In addition, the percentage of alveolar macrophages containing phagocytized CD particles was determined for each lamb.

BALF Albumin Concentration

The BALF albumin was measured as previously described (Porter at al, 20002a) by using a Cobas Fara II analyzer (Roche Diagnostic systems, Montclair, NJ), based upon its binding to bromocresol green (Doumas et al. 1991), at 658 nm using a commercial assay kit (albumin BCG diagnostic kit, Sigma Chemical Company, St Louis, MO).

BALF Lactate Dehyrogenase (LDH)

LDH activity was measured in the BALF as an indicator of cytotoxicity, by detection of the reduction of NAD^+ associated with LDH catalyzed oxidation of lactate to pyruvate at 340 nm using a commercial assay kit (Roche Diagnostics Systems, Montclair, NJ) as previously described (Porter et al, 20002a).

Alveolar Macrophage (AM) Chemiluminescence (CL)

AM CL is a marker of increased production of reactive oxygen and nitrogen species by AM. The assay was conducted in a total volume of 0.25 ml of HEPES-buffered medium as previously described (Porter et al, 20002a).

Nitric Oxide (NO) Dependent AM CL

To determine the NO-dependent chemiluminesecence released from AM in response to particle exposure, the zymosan-stimulated chemiluminescence from cells preincubated with 1 mM nitro-L-arginine methyl ester (a nonselective NOS inhibitor (Vaughan et al. 2003) was subtracted from zymozan-stimulated chemiluminescence from cells without nitro-L-arginine methyl ester as previously described (Porter et al, 20002a).

Differential Blood Count

Blood samples were collected from all sheep to monitor the changes in blood status before and after CD instillation until sacrifice. Blood collection was conducted once after sheep arrival at the pens, 1 day before instillation, and 1, 3, 5, 7, 14, 28, 42, and 56 days after instillation. Differential blood count was performed using a Cell-DYN® 3500 R (Abbot Diagnostics, Santa Clara, California). Hematocrit values were measured by using microhematocrit tubes.

Statistical Analyses

All analyses were performed with SAS version 8.2 and using Proc Mixed. In the comparison between CYP1A1 localization between AT-II and NT-II cells, in sheep exposed to BNF only, the model was the two-factor repeated measures analysis of variance. In all other cases, where data of right exposed lobe were compared to the control and to the left unexposed lobes, the model was single factor repeated measures analysis of variance. All pairwise comparisons were performed using a pooled variance estimate and Fisher's LSD (Least Significant Difference). All results were considered statistically significant at $p<0.05$.

RESULTS

1- Histopathological Changes

Histopathological changes of CD-exposed lung lobes included bronchointerstitial pneumonia with accumulation of dust-laden alveolar macrophages, mainly in the interstitial tissue with only occasional foci of alveolar histiocytosis (Fig. 1), a finding expected in a lavaged lung. These changes were irregularly distributed but consistently present in the instilled right apical lobes. Thus, bronchointerstitial pneumonia with particle-laden interstitial macrophages were observed in the right apical lobe of all instilled lambs. Bronchointerstitial pneumonia was not seen in the lung of control lambs.

2-Immunofluorescence Double Labeling for CYP1A1 and Cytokeratins 8/18

A-Effect of CD Exposure on AT-II Hyperplasia and Hypertrophy in Sheep

The area of AT-II, as indicated by lung area with green fluorescence due to cytokeratins 8/18 expression, was quantified morphometrically. AT-II hyperplasia and hypertrophy were visualized in fluorescent-stained sections of CD exposed sheep (Fig. 2). The area of AT-II was increased, albeit not significantly, in the alveolus of sheep exposed to CD and BNF compared to BNF alone (Fig. 3.).

B- Distribution of CYP1A1 in BNF-Induced Sheep.

Sheep CYP1A1 was visualized as red fluorescence in AT-II, NT-II, and endothelial cells of the alveolar septum (Fig. 2.). However, the area of CYP1A1 quantified in AT-II is significantly lower than that in NT-II of the left ($p=0.009$) and right ($p<0.001$) (Fig. 4A) apical lobes of the BNF-exposed lambs.

C- CD suppresses CYP1A1 Induction in the ovine alveolus

Morphometric quantification of the area of pulmonary alveoli expressing CYP1A1 in immunofluorescent stained sections was conducted to determine the location of CYP1A1 at the alveolar cell level.

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a- Total CYP1A1 Expression in the Entire Alveolar Septum of Sheep

The total area of CYP1A1 expression in the entire alveolar septum was significantly reduced in the right lung lobe exposed to CD and BNF compared to the left unexposed lobes or the saline-instilled right lobes of BNF control lambs (Fig. 4B.).

b- CYP1A1 Expression in AT-II

The area of CYP1A1 colocalized (co-expressed) with the AT-II marker cytokeratins 8/18 was reduced by CD exposure but differences were not statistically significant (Fig. 4C). The proportional CYP1A1 expression in AT-II, which adjusted for the increased area of AT-II, showed a highly significant reduction (68.39%) in sheep exposed to CD and BNF compared to those receiving BNF alone ($p<0.001$) (Fig. 4.). When compared to the left unexposed (internal control) lobes, the right lobes exposed to CD showed a significant diminution (63.9% reduction) of proportional CYP1A1 expression in AT-II cells ($p=0.01$) (Fig. 4D.).

c- CYP1A1 Expression in NT-II

The area of CYP1A1 expression in NT-II is significantly diminished (80.59%) in lambs exposed to CD and BNF compared to BNF alone ($p=0.0015$) (Fig. 4.) or to the left unexposed lobes of the CD-exposed sheep ($p<0.0299$) (Fig. 4E.).

3- 7-Ethoxyyresorufin-O-Deethylase (EROD) Activity

The CYP1A1-dependent enzymatic activity (EROD), was significantly reduced (31.57%) in lambs exposed to CD and BNF compared to those exposed to BNF alone (p=0.0166) or to the uninstilled lobe (left lobe) (p=0.0265) (Fig. 5A.).

4- 7-Pentoxyresorufin-O-Deethylase (PROD) Activity

The CYP2B-dependent enzymatic activity (PROD) was significantly lowered in CD-exposed lambs with BNF compared to control (p=0.042) (Fig. 5B).

5- Western Blot Analysis

CYP1A1 protein bands of right exposed lobes and left unexposed lobes were quantified and expressed as percentage of positive CYP1A1 control. The CYP1A1 apoprotein measured in lung microsomes by Western blot was reduced by 32.46% in lambs exposed to CD and BNF compared to control and reduced by 9.49% in the CD-exposed lobes compared to the control unexposed lobes, but these differences were not statistically significant (Fig. 6).

6- Results of BAL Fluid Analysis

A- BAL Cell Differentials

1- Alveolar Macrophages (AM)

The number of BAL AMs was not significantly increased by CD treatment (Fig. 7A). Cell differentials of cytospin preparation showed a significant increase (10.64%) in the percentage of AM in CD-exposed right lobes compared to the right lobes instilled with saline (p= 0.0008) and a significant increase (7.31%) compared to the unexposed left lobes (p=0.0318) (Fig. 7B). While no CD-laden AM was observed in saline exposed groups or the left unexposed lobes, an average of 44.5% of the AM counted in the BAL of CD-exposed sheep contained CD particles (range 23 to 73 %).

2- Polymorphonuclear Leucocytes (PMN)

The PMN numbers in BAL fluid were not significantly increased by CD exposure (Fig. 7C). Cell differentials of cytospin preparation exhibited a significant increase (37.7%) in the percentage of AM in CD-exposed right lobes compared to the right lobes instilled with saline (p=0.047) and a significant increase (55.5%) compared to the uninstilled left lobes (p=0.04) (Fig. 7D). This was accompanied by a decrease in the percentage of BAL lymphocytes. These results suggest that the CD-exposed lobes exhibit higher phagocytic cell percentage.

B- BAL Fluid Albumin

The albumin measured in the BAL fluid was not significantly affected by CD exposure (data not shown).

C- LDH in BAL Fluid

The LDH concentration in BAL of CD-exposed tracheal bronchial lobes was 25.6% higher than saline-exposed lobes and 17.55% higher than left uninstilled lobes (data not shown).

7- Differential Blood Count (Total Leucocytic Count)

The neutrophil, monocyte, and total leucocyte counts for both CD-exposed and control sheep were temporarily increased the day after the instillation and then gradually decreased the following days to reach almost the resting stage on the day of sacrifice

(data not shown). In addition, the hematocrit values did not show significant difference between CD-exposed sheep and controls at any time period (data not shown).

DISCUSSION

In this study, we investigated the modifying effect of respirable CD particles on CYP1A1 induction in BNF-exposed sheep. The ovine model was selected because the response of ovine lung to respirable particles, such as quartz, is similar to the human response, particularly in cellular cytotoxicity (Larivee et al. 1990). The results indicate that CD significantly inhibited CYP1A1-dependent metabolic activity (EROD) in lung microsomes (Fig. 5.). This effect was localized only in the lobes that were instilled with CD (right lobes). However, in the left lobes, which were not exposed to CD, there was no change in the activity. This suggests that CD exposure inhibited CYP1A1 metabolic activity locally and the effect did not extend to include unexposed lung lobes.

To assess the effect of CD exposure on CYP1A1 induction, the CYP1A1 protein was measured by Western blot. The amount of CYP1A1 protein measured by Western blot was reduced, albeit not significantly, in the CD-exposed right lobes compared to the saline-exposed lobes and compared to unexposed lobes. Although the reduction was not significant, the general trend seemed to be suppressive. This result suggested that CD exposure in sheep not only inhibited CYP1A1-dependent EROD activity but inhibited the CYP1A1 protein expression as well.

The suppressive effect of the CD on CYP1A1 induction in sheep lungs was further demonstrated by immunofluorescence double labeling. By using this technique, the cellular expression of CYP1A1 was investigated within the alveolus. We used cytokeratins 8/18, which are cytoskeletal proteins highly expressed in primitive epithelial cells, to recognize the AT-II (Kasper et al. 1993) in the lung alveolus. Accordingly, the cellular components of the stained lung tissue section were divided into two distinct populations using indirect immunofluorescence with a primary anti-cytokeratins 8/18 antibody and a green FITC-labeled secondary antibody. One population stained distinctively green and those were AT-II (Fig. 2A). The others did not stain green and were designated as NT-II. The area of CYP1A1 expression measured in NT-II, where no green fluorescence was visualized, was significantly higher than that area in AT-II. This result suggests that in the alveolus, AT-II are not the major sites of CYP1A1 induction and NT-II are important sites of CYP1A1 induction. In addition, there is a general deficit in the literature regarding the localization of inducible CYP1A1 in ruminant lungs. The majority of literature in ruminant CYPs concentrates on the inducibility of CYP1A1 in the liver of goat (Kasper et al. 1993) or cattle (Skalova et al. 2001) and this study is the first, to our knowledge to report the distribution pattern of CYP1A1 in sheep lungs.

The CD instillation in sheep reduced the area of CYP1A1 expression in AT-II, NT-II and the entire alveolar septum (Fig. 2D). Therefore, one mechanism of suppression of BNF-induced CYP1A1 by CD exposure appears to be inhibition of CYP1A1 expression in different alveolar septal cells. These results are not surprising and are comparable to those seen in rats exposed to CD in preliminary data (Ghanem et al. 2003). While increased size (hypertrophy) and number (hyperplasia) of AT-II were not significant in this study, they were increased numerically, which is consistent with preliminary studies in rat model (Ghanem et al. 2003). The area of CYP1A1 expression

in AT-II, relative to the total area of AT-II (proportional CYP1A1 expression, Fig. 4D) showed a significant reduction suggesting that the new hyperplastic AT-II do not express CYP1A1 in proportion to their number and size. This result suggested that CD exposure led to production of a new population of AT-II with decreased CYP1A1 expression. The mechanism of downregulation of CYP1A1 associated with cellular proliferation should be further investigated. However, in the rat liver with hyperplastic nodules, induced by diethylnitrosamine and partial hepatectomy, the total amount of microsomal CYP enzymes was reduced 50% compared to the control (Degawa et al. 1995). Moreover, the inducibility of CYP1A by inducers decreases slightly in the rat liver bearing hyperplastic nodules (Degawa et al. 1995). Consistent with that, the inducibility of CYP1A1 by BNF was markedly reduced in early lung hyperplastic foci associated with urethane exposure and the lung carcinomas were devoid of expression of CYP1A1 protein (Forkert et al. 1998). All of these previous studies suggest that CYP protein is downregulated in proliferating cells, a finding, which is consistent with the downregulation of CYP1A1 induction and CYP2B in our study of CD-exposed lambs.

Along with CYP1A1 activity, the activity of another CYP isoform, CYP2B, was measured. CYP2B is the major constitutive isoform of CYP family in sheep lungs (Williams et al. 1991). The CYP2B-dependent enzymatic activity (PROD) showed a significant diminution in lung microsomes prepared from lung of sheep exposed to CD and BNF compared to BNF alone (Fig. 5B.). This result suggested that, CD not only inhibited the activity of BNF-induced CYP1A1 in sheep lung, but also the activity of another CYP isoform, CYP2B, which is constitutively expressed in sheep lungs.

To assess the local inflammatory reaction of sheep lung in response to the inhaled CD particles, the AM and PMN were counted in BAL. The percentage of AM and PMN (Fig. 7) showed a modest, but significant increase. This result suggested that an inflammatory process accompanied the CD instillation in sheep (pneumoconiosis). The local inflammatory process was also associated with non-significant enhancement of LDH (marker of cytotoxicity) and albumin levels (marker of pulmonary-blood barrier damage in the lung) of the CD-exposed group compared to control. Interstitial pneumonia with accumulation of dust-laden AM in the interstitial tissue were the hallmarks of histopathological changes in lung sections of CD-exposed lobes in sheep (Fig. 1.). In spite of the local inflammatory reaction, no systemic reaction was observed as there was no significant change in the total leucocyte count, neutrophil count, or monocyte count between CD-exposed and control sheep. This result suggested that CD instillation into a localized region of the lung was not associated with a systemic reaction in lambs at the dose instilled (500 mg/sheep). This suggested that CD exposure in sheep is associated with an inflammatory response, which involves the interstitium consistently. Previous studies involving liver or *in vitro* systems demonstrated an association between inflammation and decreased activity of different CYP isoforms, such as CYP1A, CYP2A, CYP2B, CYP2D9, CYP3A, CYP2E1 and CYP4A (Warren et al. 1999; Siewert et al. 2000; Jover et al. 2002; Carcillo et al. 2003). Moreover, some other studies suggest that CYP2B1 is associated with diesel exhaust particles and carbon black in rat lung (Rangasamy et al. 2003). In our study, we have a consistent association of suppression of pulmonary CYP1A1 and CD exposure in an ovine model.

The response of the rat lung to high concentrations of respirable particles is of debatable relevance for human risk assessment (ILSI, 2000). In particular, the alveolar

epithelial cell proliferation seen in response to respirable particles is more pronounced in rats than in primates (Nikula et al. 1997). For that reason, an ovine model was used to investigate the effects of CD on CYP1A1 induction in the lung. We found that CD suppressed CYP1A1 induction. In addition, the activity of the major constitutive isoform of CYP in the ovine lung, CYP2B, was suppressed by respirable CD. This is similar to suppression of CYP2B1 previously noted in the particle-exposed rat lung (Ma and Ma, 2000).

In conclusion, CD suppressed the activity of both CYP1A1 and CYP2B in a non-rodent, ovine model. This is consistent with the inflammation-associated downregulation of many CYP isoforms in rodent model systems.

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Figure Legends

Fig.1. Histopathological changes in CD-exposed lobes. A. Bronchointerstitial pneumonia with alveolar and interstitial accumulation of dust-laden AM in a CD-exposed lobe (bar = 50 µm). B. A higher magnification of PA region showing aggregates of dust-laden macrophages in the interstitium (white arrow heads). AT-II hyperplasia is also shown (black arrow heads) Bar = 50 µm.

Fig. 2. Immunofluorescent images showing the suppression of CYP1A1 expression in the alveolus after CD and BNF exposure. A. Immunofluorescent imaging of the ovine lung treated with BNF alone showing the general distribution pattern of cytokeratins 8/18 (green) which is expressed primarily in AT-II that are cuboidal in shape and located at the corners of alveolar septa. Cells that do not express cytokeratins 8/18 are not green and are usually elongated cells (NT-II) B. Immunofluorescent imaging of the ovine lung treated with BNF alone showing the general distribution pattern of CYP1A1 (red). C. Dual immunofluorescence for CYP1A1 (red) and cytokeratins 8/18 (green) in the uninstilled left lobe of a BNF-injected sheep showing that most of the CYP1A1 is localized in NT-II that do not express cytokeratins 8/18. CYP1A1 expression in AT-II is

indicated by a yellow color. D. Using dual immunofluorescence, the red immunofluorescence for CYP1A1 is reduced throughout the alveolar septum in the CD-exposed right lung lobe. Bar=20 µm.

Fig. 3. Morphometric quantification of the cytokeratins 8/18 expression in alveoli of CD exposed vs. control sheep. The area of cytokeratins 8/18 expression is higher, albeit not significantly, in the CD-exposed right lung lobes of lambs treated with CD and BNF compared to the left unexposed internal control lobes and compared to right lobes of control BNF-treated lambs. Results represent means ± SE, n=5 in the saline-exposed group and 4 in the CD-exposed group.

Fig. 4. Morphometric quantification of CYP1A1 expression in the ovine pulmonary alveolus. A. Morphometric analysis showing the distribution of CYP1A1 in ovine lung. The area of CYP1A1 expression in NT-II is significantly larger compared to CYP1A1 area in AT-II of left and right lung lobes. B. The area of CYP1A1 expression in the alveolus of the CD-exposed right lung lobe of lambs treated with BNF is significantly lower (74.14%) than with BNF alone and is reduced by 65.3 % compared to the left unexposed lobes of CD-exposed sheep. C. The area of CYP1A1 expression in AT-II was reduced, albeit not significantly in CD-exposed lobes compared to controls. D. The proportional CYP1A1 expression in AT-II of BNF-induced lambs is significantly reduced in the CD-exposed right lung lobe compared to the left unexposed lobes and the right lobe of lambs receiving only BNF. E. Morphometric quantification of the area of CYP1A1 expression in NT-II. In BNF treated lambs, the area of CYP1A1 expression in NT-II is significantly lower in the right CD-exposed lobes compared the left unexposed lobes of the same lambs or the unexposed lung of lambs receiving BNF alone. Results represent means ± SE, n=5 in the saline-exposed group and 4 in the CD-exposed group. * indicates significant difference at $p<0.05$. + indicates significant difference at $p<0.001$.

Fig. 5. Effect of CD exposure on the EROD and PROD activity in BNF-exposed lambs. A. In BNF-induced lambs, EROD is significantly reduced in the CD-exposed right lung lobes compared to the left unexposed lobes or the lung of lambs receiving BNF alone. B. PROD is significantly reduced in lambs exposed to CD and BNF compared to lambs with BNF alone and is reduced 53.45 %, albeit not significantly, in CD-exposed lobes compared to the left unexposed lobes. Results are means ± SE, n=5 in the saline-exposed group and 4 in the CD-exposed group. * indicate Significant difference at $p<0.05$.

Fig. 6. Western blot analysis showing the reduction of CYP1A1 immunoprotein by CD exposure. A. western blot for the left unexposed lobes. B. Western blot for the right exposed lobes. C. Graphical representation of the amount of CYP1A1 protein measured by Western blot. The letter c above the lane means control. MW means molecular weight standard. Lanes from 1 to 4 are for saline-exposed lambs (control) while lanes 6 to 10 are for CD-exposed lambs.

Fig. 6. Differential count of ovine BAL cells. A. The AM yield is not significantly higher in CD-exposed right apical lobe than saline-exposed lobe or the left uninstilled lobes. B. The percentage of AM in BAL cytospin is significantly increased in CD-exposed right

apical lobes than saline-exposed lobes or the left unexposed lobes. C. The PMN yield is not significantly higher in CD-exposed lobes than saline-exposed lobes and the left unexposed lobes. D. the percentage of PMN in BAL cytospin is significantly higher in CD-exposed lobes than saline-exposed lobes and the left unexposed lobes. Results represent means ± SE, n=5 in the saline exposed group and 4 in the CD-exposed group. * indicates significant difference at $p<0.05$.

Figure 1 histopathology

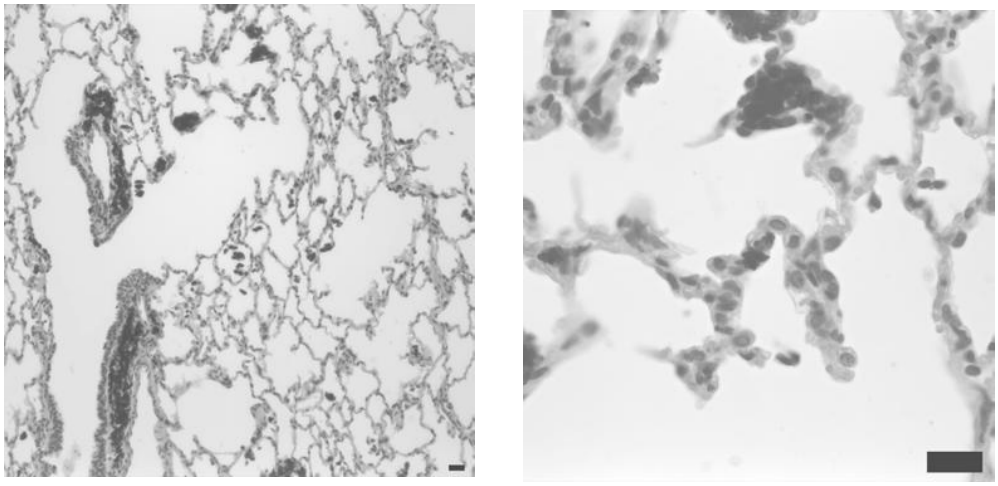
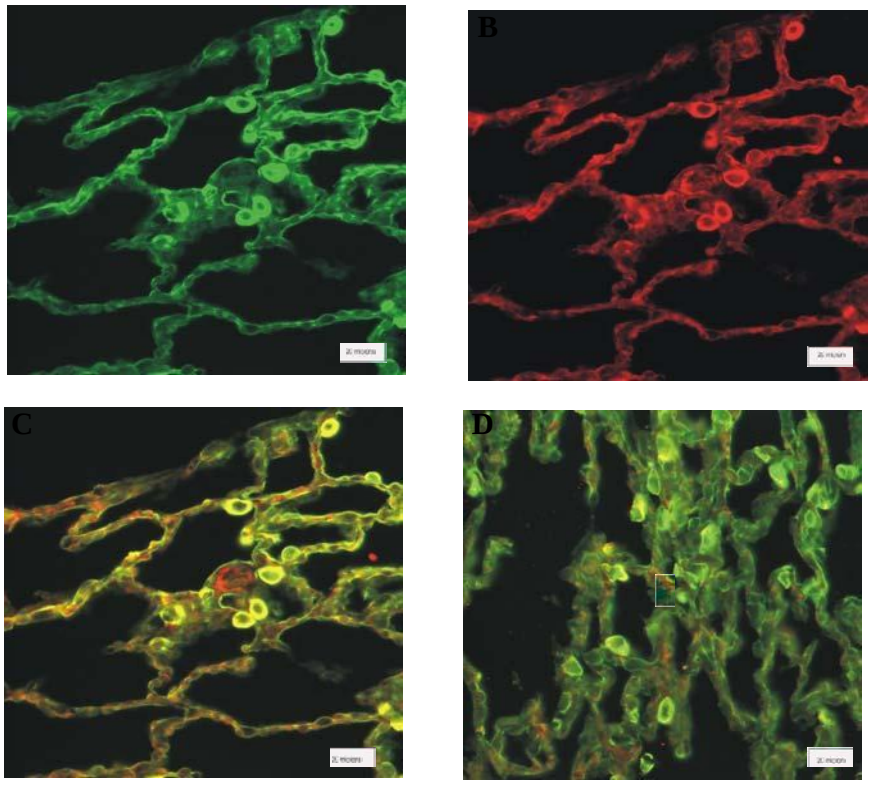


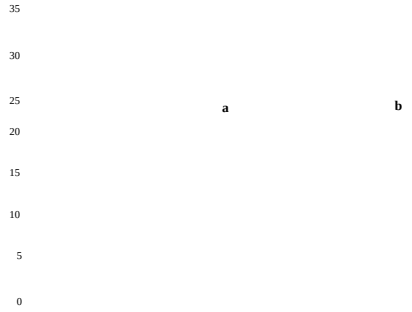
Figure 2



E

Figure 5

A



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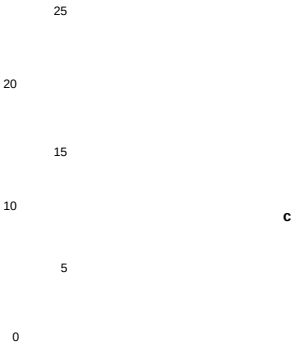
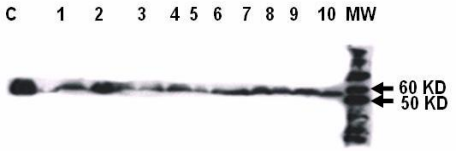


Figure 6

A



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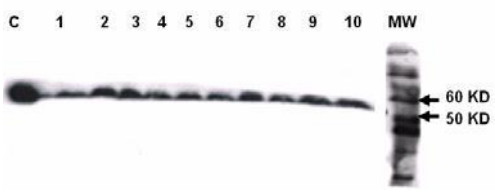

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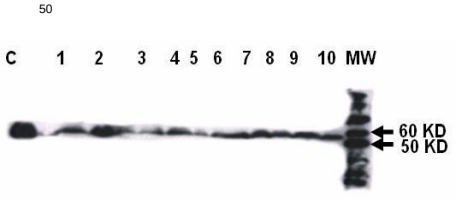
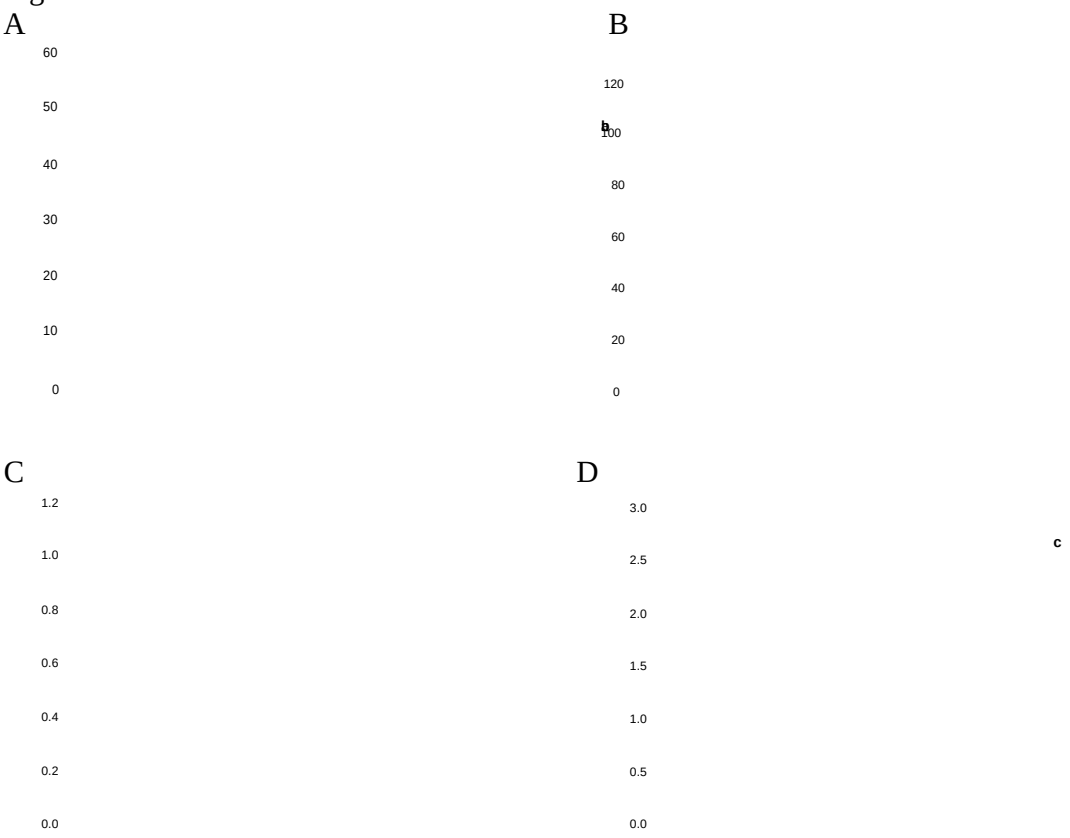


Figure 7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