Radical-Containing Ultrafine Particulate Matter Initiates Epithelial-to-Mesenchymal Transitions in Airway Epithelial Cells

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Environmentally persistent free radicals (EPFRs) in combustion-generated particulate matter (PM) are capable of inducing pulmonary pathologies and contributing to the development of environmental asthma. *In vivo* exposure of infant rats to EPFRs demonstrates their ability to induce airway hyperresponsiveness to methacholine, a hallmark of asthma. However, the mechanisms by which combustion-derived EPFRs elicit *in vivo* responses remain elusive. In this study, we used a chemically defined EPFR consisting of approximately 0.2 μm amorphous silica containing 3% cupric oxide with the organic pollutant 1,2-dichlorobenzene (DCB-230). DCB-230 possesses similar radical content to urban-collected EPFRs but offers several advantages, including lack of contaminants and chemical uniformity. DCB-230 was readily taken up by BEAS-2B and at high doses (200 $\mu\text{g}/\text{cm}^2$) caused substantial necrosis. At low doses (20 $\mu\text{g}/\text{cm}^2$), DCB-230 particles caused lysosomal membrane permeabilization, oxidative stress, and lipid peroxidation within 24 hours of exposure. During this period, BEAS-2B underwent epithelial-to-mesenchymal transition (EMT), including loss of epithelial cell morphology, decreased E-cadherin expression, and increased α -smooth muscle actin (α -SMA) and collagen I production. Similar results were observed in neonatal air-liquid interface culture (i.e., disruption of epithelial integrity and EMT). Acute exposure of infant mice to DCB-230 resulted in EMT, as confirmed by lineage tracing studies and evidenced by coexpression of epithelial E-cadherin and mesenchymal α -SMA proteins in airway cells and increased SNAI1 expression in the lungs. EMT in neonatal mouse lungs after EPFR exposure may provide an explanation for epidemiological evidence supporting PM exposure and increased risk of asthma.

Keywords: particulate matter; epithelial-mesenchymal transition; environmental asthma; pediatric

Combustion-generated particulate matter (PM) from industrial processes and burning of biomass and fossil fuels has been linked with adverse pulmonary health effects (1). Environmental PM, both fine and ultrafine, is capable of airway deposition, alveolar penetration, respiratory distress, and exacerbation of preexisting

CLINICAL RELEVANCE

Environmentally persistent free radicals (EPFRs) in combustion-generated particulate matter (PM) are capable of inducing pulmonary pathologies and possibly contributing to the development of environmental asthma. Acute inhalation exposure of infant mice to EPFRs resulted in epithelial-to-mesenchymal transition (EMT) in the lungs. EMT in neonatal mouse lungs after EPFR exposure may provide an explanation for epidemiological evidence supporting PM exposure and increased risk of asthma.

pulmonary conditions. Previous studies highlight the potential roles of PM exposure in predisposing to asthma and pulmonary fibrosis (2–4). Additionally, PM has adjuvant effects when combined with innocuous antigen (5–7) and induces cellular damage, stimulating fibrotic remodeling in adult rodent exposure models (2). The developing pulmonary and immune systems are particularly vulnerable (8). We have developed a model for studying particulate exposures in neonatal rodents (< 7 d of age) (9), which we apply here to understand the effects of combustion-generated environmentally persistent free radicals (EPFRs) on pulmonary airway remodeling.

Delineation of the influences of particulate burden from the reactive chemical species complexed with the particulate has proven difficult. The nature of the chemical species drastically influences the cellular response (10), thus complicating linking epidemiology data to a toxicological or immunological exposure mechanism (11, 12). By extension, the link between PM exposure and predisposition to pulmonary conditions, including asthma, has remained elusive (13). To understand the health effects of PM, we have focused on combustion-generated EPFRs in the ultrafine (0.2 μm) range. These EPFRs have been identified in airborne PM (14–16) and in soils at abandoned hazardous waste facilities that fall under the Comprehensive Environmental Response, Compensation, and Liability Act of 1980, termed Superfund sites (17). These species consist of phenoxyl- and semiquinone-type radicals formed and stabilized by transition metal oxide-containing particles (18), forming a pollutant-particle system that acts as an integrated entity. This pollutant-particle system induces oxidative stress in biological systems (16) and participates in Fenton-like cyclic chain reactions without significant destruction of the original EPFR (19, 20). We have modeled EPFRs in a laboratory combustion setting (16). Our model pollutant-particle system, which we refer to as DCB-230, consists of 1,2-dichlorobenzene (1,2-DCB or DCB) chemisorbed onto a silica/Cu(II)O substrate (a metal oxide commonly found in biomass) via a combustion process at 230°C. The chemical, 1,2-DCB, is

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commonly used in industrial processes and can generate the intermediate chlorobenzene (21). A reaction between the organic compound and the transition metal oxide forms a surface-associated, stabilized free radical (14).

Our recent *in vivo* studies using DCB-230 have shown considerable induction of pulmonary inflammation in neonatal rats (9). This correlated with increased oxidative stress, inflammatory cytokine production, and airway hyperresponsiveness. Pulmonary pathology showed substantial increases in smooth muscle mass and suggested significant airway remodeling as the primary culprit of lung dysfunction. Particulate controls lacking the EPFR did not induce airway remodeling or collagen deposition, implicating the EPFR in the observed pulmonary response. Proteomic analyses of the neonatal lung after exposure to DCB-230 revealed an increase in a protein cofilin-1 (9), a protein involved in actin depolymerization and epithelial-to-mesenchymal transition (EMT) (22) and implicated in steroid-insensitive asthma in humans (23). We therefore hypothesized that DCB-230 initiates EMT, which may be the underlying factor contributing to persistent airway hyperresponsiveness. We investigated the influences of DCB-230 on cell viability and monitored changes in epithelial cell morphology and expression related to EMT *in vitro* using cell lines and confirmed the EMT phenotype in primary neonatal airway epithelial cells in the developing neonatal lung. We demonstrate that DCB-230 induces oxidative stress, which drives EMT in lung epithelium.

MATERIALS AND METHODS

Additional methods are provided in the online supplement.

Combustion-Generated EPFR-Containing PM

The EPFR-containing PM, DCB-230, was fabricated (16) and characterized (9). The total EPFR concentration was 2.5 $\mu\text{M/g}$ PM, and mean diameter was less than 0.2 μm (16). Immediately before use, DCB-230 was suspended in irrigation saline containing 0.02% Tween 80 as previously described (24). *In vitro* exposures used 20 and 200 $\mu\text{g}/\text{cm}^2$ doses, whereas *in vivo* inhalation exposures used an environmentally relevant dose of 200 $\mu\text{g}/\text{m}^3$.

Cells and Animals

BEAS-2B cells and mouse neonatal airway epithelial cells (25) were used for the *in vitro* studies.

Brown-Norway rat or C57BL/6 pups were used for *in vivo* studies and mated to produce pups. Pups (4 d of age) were exposed to aerosolized DCB-230 (200 $\mu\text{g}/\text{cm}^2$) for 30 min/d for 7 days. Clara cell-specific, codon-improved Cre (CCSP-iCre) mice were developed by and obtained from Dr. Francesco DeMayo and were bred to R26Rosa-lox-Stop-lox-LacZ mice (Jackson Labs, Bar Harbor, ME). Animal protocols were approved by the Institutional Animal Care and Use Committee at the Louisiana State University Health Sciences Center.

RESULTS

Exposure of Bronchiolar Epithelial Cells to Combustion-Generated EPFR-Containing Particles Induces Phenotypic Changes Consistent with EMT

From our previous studies with EPFR-containing particles, it was unclear if DCB-230 was actively taken into cells where it could directly affect cell physiology, redox-sensitive signaling pathways, and function. We used our previous data (14, 15) and published reports (26–28) to establish 20 $\mu\text{g}/\text{cm}^2$ as a target dose with environmental relevance, resulting in a viability of $40.1 \pm 1.8\%$ (see Figure E1 in the online supplement). BEAS-2B cells were exposed to vehicle or DCB-230 at 20 $\mu\text{g}/\text{cm}^2$ for 1 to 6 hours and analyzed by TEM to visualize uptake (Figure 1A). DCB-230 aggregation on the cell membrane and uptake was observed within 3 hours after exposure and by 6-hour aggregates were prominently visible inside elongated epithelial cells.

We next sought to characterize the phenotypic differences observed by TEMs between DCB-230 and vehicle-exposed cells. BEAS-2B cells exposed to DCB-230 (20 $\mu\text{g}/\text{cm}^2$) were stained to visualize F-actin and lysosomes at 0, 3, 6, and 24 hours (Figure 1B). Unexposed cells displayed the prototypical epithelial cobblestone morphology, with F-actin localized mostly at the cell periphery and weak lysosome staining (data not shown; cells imaged before DCB-230 at 0 h shown for reference). As the DCB-230 exposure period increased, cells disengaged from neighboring cells, and a substantial increase in lysosomal staining was observed. Between 3 and 6 hours, cells became elongated with prominent F-actin staining and perinuclear clouding of lysosomes, suggesting increased phagocytosis and oxidative stress. Unexposed cells continued to display a cobblestone morphology, with F-actin mostly localized to the cell periphery. At 6 hours, F-actin staining was more prominent inside exposed cells and organized along the primary axis of the cells. This was accompanied

Figure 1. Exposure of BEAS-2B cells to 1,2-dichlorobenzene (DCB-230) induces phenotypic changes characteristic of epithelial-to-mesenchymal transition. (A) BEAS-2B cells were exposed to vehicle (0.9% saline, 0.02% Tween-80) or DCB-230 (20 $\mu\text{g}/\text{cm}^2$) for 6 hours and imaged using transmission electron microscopy. Low-magnification images of cells exposed for 6 hours show membrane invaginations forming around an aggregate of DCB-230 particles (arrows). Scale bars, 1 μm . (B) Cytoskeletal changes were recorded over a 24-hour exposure period by staining for F-actin (Alexa Fluor 488-phalloidin; green) and lysosomes (Lyso-tracker; red). Within 6 hours of exposure, F-actin polymerization and a morphology change from cobble-stone to spindle was readily apparent in the cells exposed to DCB-230 as compared with controls. Increased actin polymerization observed at 6 hours after exposure correlated with an increase in perinuclear lysosome accumulation. Scale bars, 10 μm . (C) Levels of the cofilin-1 (CFL-1) were determined by Western blot ($n = 2$) 24 hours after vehicle or DCB-230 exposure and (D) quantified by densitometry. Values represent mean $\pm$ SEM.

by retraction of cells from neighboring cells and the adoption of a more spindled-shaped morphology. By 24 hours, exposed cells had lost their cobblestone morphology and adopted an elongated spindle-shaped morphology characteristic of mesenchymal cells with prominent actin fibers throughout the cell. Vinculin and F-actin were coexpressed along the spindled axes of exposed cells, suggesting modulation of focal adhesions (data not shown). To further investigate changes in actin dynamics and focal adhesions, Western blotting was performed for cofilin-1 in protein extracts from DCB-230-exposed or vehicle-exposed cells at 24 hours after exposure. We observed an increase in expression of cofilin-1 with exposure to DCB-230 (Figures 1C and 1D).

DCB-230 Uptake Causes Lysosomal Membrane Permeabilization, Oxidative Stress, and Lipid Peroxidation

We hypothesized that the changes in cell morphology were due to oxidative stress induced by DCB-230. Lysosomal membrane permeabilization (LMP) resulting in particle liberation into the cytosol has previously been shown to initiate cascades of intracellular responses. To examine this possibility, we incubated cells with acridine orange (AO) and monitored protonation of the dye using microscopy until perinuclear lysosomes were evident. We then monitored decreases in the mean fluorescent intensity (MFI) of AO by flow cytometry. Perinuclear clouding of vesicles containing slightly protonated AO were visible within 1.5 hours of exposure. By 3 hours, the lysosomes stained orange-red, consistent with AO protonation, and were located predominantly in the perinuclear region (Figure 2A). Given this finding, we examined MFI at 3 and 6 hours after vehicle or DCB-230 exposure (Figures 2B and 2C). No significant decrease in MFI was observed at 3 hours; however, by 6 hours a significant left shift in the MFI was observed (Figure 2C), suggesting LMP.

Because exposure to DCB-230 led to LMP in bronchiolar epithelial cells, we next measured the ratio of reduced to oxidized glutathione (GSH/GSSG) as a measure of oxidative stress (Figure 2D). GSH/GSSG ratios were quantified in vehicle- or DCB-230-exposed cells at 6 hours (i.e., at the time of DCB-230 uptake

and LMP) and 24 hours (i.e., at the time of decreased cell viability and altered morphology). At 6 hours, GSH/GSSG ratios were reduced to 2.6 ± 0.8 from 27.9 ± 4.9 in vehicle-exposed cells. By 24 hours, the GSH/GSSG ratio in DCB-230-exposed cells had not recovered compared with vehicle-exposed cells (3.8 ± 0.3 versus 34.4 ± 7.3).

Because LMP and oxidative stress were observed, we hypothesized that oxidative end-products, such as lipid peroxidation, would be present in culture supernatant from exposed cells. Culture media was sampled from vehicle or DCB-230-exposed cells and assayed for 8-isoprostanes (8-IP) (Figure 2D). By 24 hours, a significant increase in 8-IP in the media of DCB-230-exposed cells (84.4 ± 12.2 pg/ml) was observed compared with vehicle-exposed cells (50.2 ± 6.6 pg/ml).

BODIPY incorporates into lipid membranes and, upon oxidation, undergoes a spectral shift from red to green. In some cells exposed to DCB-230, a significant increase in green fluorescence could be observed in the perinuclear region 6 hours after exposure (Figure 2E). This staining was consistent with the location of acidic vesicles using LysoTracker (Figure 1B) and acridine orange (Figure 2A) and appeared to be organelle associated. Vehicle-exposed cells continued to emit only red fluorescence, suggesting that intracellular lipid peroxidation occurred only in the presence of DCB-230.

DCB-230 Alters Epithelial Cell Morphology Consistent with an EMT

The increased smooth muscle mass observed *in vivo* along with oxidative stress and changes in cell morphology observed *in vitro* led us to hypothesize that bronchiolar epithelial cells were undergoing EMT. During EMT, epithelial cells lose E-cadherin (E-Cad) junctions while increasing production of α -smooth muscle actin (α -SMA) and collagen I. In addition, change to mesenchymal lineage is accompanied by dynamic changes in the cytoskeleton, which can be observed as a remodeling of focal adhesions (phosphorylation of focal adhesion kinase [pFAK]). To investigate several genes involved in EMT,

Figure 2. DCB-230 uptake induces lysosomal membrane permeabilization, oxidative stress, and lipid peroxidation. (A) Lysosome acidification and permeabilization were monitored using acridine orange (AO). Epithelial cells preloaded with AO and exposed to DCB-230 were imaged hourly to monitor lysosome acidification (orange perinuclear stain). During the exposure period, morphological changes including cell dissociation (arrows) were apparent. (B) Lysosome destabilization was monitored by recording mean fluorescent intensity (MFI) of AO in the phycoerythrin channel by flow cytometry. Representative histogram plots are shown for

vehicle-treated (solid gray) and DCB-230-treated (black line) cells at 3 and 6 hours. (C) MFI of AO decreased significantly in DCB-230-exposed cells after 6 hours of exposure ($*P < 0.05$; $n = 3$), indicating acidification. (D) Oxidative stress was quantified by plotting the ratio of reduced glutathione (GSH/GSSG). The GSH/GSSG ratio was significantly decreased in cells exposed to DCB-230 at 6 and 24 hours ($*P < 0.05$; $n = 3$). Lipid peroxidation was quantified by ELISA for 8-isoprostane. 8-Isoprostane significantly increased in cell culture supernatants at 24 hours after exposure ($*P < 0.05$; $n = 3$). (E) Lipid peroxidation was visualized as early as 6 hours after exposure using BODIPY 581/591 C11 and observed as an increase in perinuclear fluorescence (green). Values represent mean $\pm$ SEM. Scale bars, 10 μ m.

an 84-gene EMT PCR array was performed on BEAS-2B cells exposed to vehicle or DCB-230 for 24 hours. Fold changes in genes involved in cell–cell junctions, cytoskeletal remodeling, extracellular matrix production, and TGF- β signaling pathway were analyzed. The junction proteins E-Cad (*CDH1*), occludin (*OCN*), notch 1 (*NOTCH1*), β -catenin (*CTNNB1*), and desmoplakin (*DSP*) were down-regulated in the DCB-230-exposed cells. This coincided with increased expression of several repressors of E-Cad, including snail homolog 1 (*SNAIL*), SLUG (*SNAI2*), SMUC (*SNAI3*), transcription factors 3 (*TCF3*) and -4 (*TCF4*), and zinc finger E-box binding homeobox 1 (*ZEB1*) and -2 (*ZEB2*). Increased expression of matrix metalloproteinases (*MMP3*, *MMP9*) was also observed (see Table E1 in the online supplement).

To verify these changes, we selected key genes involved in EMT and evaluated their expression in BEAS-2B cells 24 hours after DCB-230 exposure using TGF- β as a positive control (Figure 3A). Compared with vehicle-treated cells, DCB-230 exposure resulted in decreased levels of E-CAD expression at the

cell periphery and, similar to TGF- β , increased expression of α -SMA, pFAK, and collagen I. To further validate an EMT phenotype, we probed for mRNA expression of E-Cad (*CDH1*), α -SMA (*ACTA2*), and *SNAIL* (Figure 3B). Exposure to DCB-230 resulted in a significant decrease in *CDH1* expression (0.4 ± 0.1 -fold), a trending increase in *ACTA2* (3.7 ± 1.2 -fold), and a significant increase in *SNAIL* expression (10.2 ± 2.1 -fold) compared with vehicle-exposed cells.

Because decreased GSH/GSSG ratios and increased 8-IP levels after DCB-230 exposure suggested oxidative stress and because oxidative stress correlated with EMT, we interrogated an oxidative stress gene array to identify candidate genes involved in the antioxidant response to DCB-230 (data not shown). The PCR array demonstrated that superoxide dismutase 2 (*SOD2*) was highly up-regulated in epithelial cells after exposure to DCB-230. We validated this result at the mRNA and protein levels. *SOD2* mRNA was up-regulated 3.3 ± 0.5 -fold in BEAS-2B-exposed cells compared with vehicle (Figure 3B). Western blot was performed on cell lysates and confirmed that *SOD2* protein was also

Figure 3. DCB-230 induces phenotypic changes characteristic of epithelial-to-mesenchymal transition. (A) Representative immunohistochemistry images of expression of epithelial and mesenchymal markers in vehicle or DCB-230-exposed BEAS-2B cells at 24 hours of exposure or in TGF- β -exposed (2.5 ng/ml for 6 d, positive control) BEAS-2B cells (all markers green and nuclei counterstained with DAPI). Exposure to DCB-230 dramatically reduces expression of epithelial markers (E-cadherin [E-Cad]) and dramatically increases expression of mesenchymal markers (α -smooth muscle actin [α -SMA], phosphorylated focal adhesion kinase [pFAK], and collagen I) compared with vehicle-treated cells. In DCB-230-exposed cells, which had undergone morphological changes due to exposure, E-Cad staining relocated from the interface between cells to the cytoplasm, and α -SMA, punctate pFAK staining, and peripherally localized collagen I became apparent. Little to no expression of α -SMA, pFAK, or collagen I was observed in vehicle-exposed cells. TGF- β induced epithelial and mesenchymal marker expression and localization similar to DCB-230 exposure. (B) Expression of E-Cad (*CDH1*), α -SMA (*ACTA2*), snail homolog 1a (*SNAIL*; a zinc finger transcriptional repressor that mediates EMT),

and superoxide dismutase 2 (*SOD2*; an enzyme important in reducing mitochondrial superoxide) was evaluated in exposed cells. Fold change in gene expression (relative to vehicle) was plotted after normalization to the housekeeping gene hypoxanthine phosphoribosyltransferase 1. DCB-230 exposure significantly decreased *CDH1* and significantly increased *SNAIL* and *SOD2* (* $P < 0.05$; $n = 3$). (C) Changes in α -SMA and *SOD2* were verified by Western blot of protein lysates with GAPDH as loading control ($n = 3$). Values represent mean $\pm$ SEM. Scale bars, 10 μ m.

increased in conjunction with an increase in α -SMA (Figure 3C), both of which also correlated to EMT phenotype observed by IHC.

SOD2 Supplementation Reverses Select EMT Gene Expression in DCB-230-Exposed Epithelial Cells

SOD2 protects from mitochondrial oxidative stress by converting superoxides to H_2O_2 and O_2 . Because exposure to DCB-230 increased SOD2 expression, we hypothesized that intracellular levels of H_2O_2 would be significantly higher in exposed cells. After normalizing to total protein, H_2O_2 levels were measured in cell lysates exposed to vehicle or DCB-230 for 24 hours using Amplex Red (Figure 4A). We observed a significant increase in intracellular H_2O_2 in the lysates from cells exposed to DCB-230 compared with vehicle ($181.0 \pm 17.3 \mu\text{M}/\text{mg}$ versus $109.7 \pm 4.8 \mu\text{M}/\text{mg}$). We hypothesized that supplementing SOD2 enzyme may protect epithelial cells from EMT by increasing antioxidant potential. We first compared the effects of non-cell-permeant SOD2 (SOD) and cell-permeable SOD2 (MnTMPyP) on H_2O_2 levels (Figure 4B). SOD2 supplementation before DCB-230 exposure significantly decreased H_2O_2 levels ($119.4 \pm 5.2 \mu\text{M}/\text{mg}$), whereas MnTMPyP significantly increased (497.4 ± 32.5) H_2O_2 levels compared with no supplementation controls (Figure 4B; compare with *dashed lines* on graph). Expression of key EMT genes was evaluated in SOD- and MnTMPyP-supplemented cells exposed to vehicle or DCB-230 (Figure 4C). SOD and MnTMPyP significantly increased *CDH1* expression (1.0 ± 0.3 - and 4.3 ± 1.3 -fold) and decreased *SNAIL* expression (1.6 ± 0.4 - and 3.2 ± 0.1 -fold) compared with DCB-230-exposed cells without supplementation (0.4 ± 0.1 - and 10.2 ± 2.1 -fold). *ACTA2* expression was only significantly decreased in SOD-treated, DCB-230-exposed

cells (1.6 ± 0.7 -fold and 3.7 ± 1.2 -fold). SOD significantly decreased *SOD2* expression (1.2 ± 0.2 -fold), whereas MnTMPyP increased *SOD2* expression (6.8 ± 1.3 -fold) compared with DCB-230-exposed cells without supplementation (3.3 ± 0.5 -fold).

DCB-230 Induced EMT in Primary Neonatal Airway Epithelial Cells Cultured at the Air-Liquid Interface

We next examined whether an EMT phenotype occurred in primary neonatal airway epithelial cells cultured at the air-liquid interface (ALI). ALIs were exposed to vehicle or DCB-230 for 24 hours. Vehicle exposure led to no visible disruption in the epithelial layer (Figure 5A). Exposure to DCB-230 led to significant disruption of the epithelial layer, characterized by the presence of dense patches of cells at the injury margin and spindle-shaped cells predominantly within the injured epithelial layer. In cross-sections of vehicle-exposed ALIs, the cell monolayer was preserved, whereas DCB-230-exposed ALIs had significant shedding of the uppermost layer and an increase in the abundance of cells with a spindle-shaped morphology through the injured area. To verify EMT, inserts were stained with E-cad and α -SMA and imaged at the insert center for vehicle treated or at the site of injury for DCB-230 (Figure 5B). Cells coexpressing α -SMA and E-cad were observed only in the monolayers exposed to DCB-230. Coexpression was fairly homogenous at the site of injury and heterogeneous at the edges of the injury. High-magnification images show α -SMA fibers in cells undergoing EMT.

To quantitatively demonstrate dysfunction of the epithelial monolayer, permeability to conjugated dextran was determined

Figure 4. SOD2 supplementation reverses expression of EMT markers in DCB-230-exposed epithelial cells. SOD2 converts superoxides to H_2O_2 . (A) Intracellular levels H_2O_2 were measured in cells exposed to vehicle or DCB-230 for 24 hours using Amplex Red. Results are represented as μM H_2O_2 per mg total protein. DCB-230 exposure significantly increased intracellular H_2O_2 ($*P < 0.05$; $n = 4$). (B) To aid detoxification of DCB-230-induced superoxide production, cells were supplemented with a cell-impermeable and a cell-permeable superoxide dismutase (SOD and MnTMPyP, respectively). SOD significantly decreased and MnTMPyP significantly increased H_2O_2 levels compared with no supplementation (*dashed lines*). (C) Expression of EMT panel genes were evaluated in SOD- and MnTMPyP-supplemented cells exposed to vehicle or DCB-230. Fold change in gene expression (relative to treatment-matched vehicle group) was plotted after

normalization to *HPRT*. Expression levels were then compared between DCB-230-exposed cells with or without SOD/MnTMPyP supplementation ($*P < 0.05$ versus DCB-230 alone; $n = 4$). SOD and MnTMPyP significantly increased *CDH1* expression and decreased *SNAIL* expression, whereas only SOD decreased *ACTA2* expression. SOD significantly decreased *SOD2* expression, whereas MnTMPyP increased *SOD2* expression. Values represent mean $\pm$ SEM.

Figure 5. DCB-230 induced EMT in primary neonatal airway epithelial cells cultured at the air–liquid interface (ALI). (A) Disturbance of the epithelial monolayer after 24-hour exposure to DCB-230 was observed by hematoxylin and eosin staining of fixed whole inserts (image of apical surface; scale bar, 100 μ m) and insert cross-sections (A: apical surface side; scale bar, 10 μ m). (B) EMT phenotype in neonatal ALI after exposure was verified by E-cad (red) and α -SMA (green) staining of insert cross-sections. Reference images were taken of the cross-sectional center for vehicle-treated and injured epithelial monolayer for DCB-230. Scale bars, 10 μ m. Inset images demonstrate cells positive for E-cad only in vehicle exposure and positive for E-cad and α -SMA after DCB-230 exposure. (C) Loss of epithelial integrity was quantified by FITC-dextran

permeability after 24 hours of exposure to vehicle or DCB-230. Values were plotted as percent change in dextran content in the basal compartment with respect to baseline and represent mean $\pm$ SEM (* P < 0.05; n = 6). (D) Expression of EMT markers in RNA extracted from cells after 24 hours of exposure to vehicle or DCB-230 by real-time quantitative PCR. *Hprt1* was used as housekeeping gene, and fold expression was determined using the delta-delta Ct method. DCB-230 exposure significantly increased expression of *Cdh1*, *Acta1*, and *Snail*. Values represent mean $\pm$ SEM (* P < 0.05; n = 3).

after 24 hours of exposure (Figure 5C). DCB-230 exposure caused an increase in permeability relative to vehicle-exposed cells. EMT markers in neonatal ALI cultures were assessed by quantifying fold change in expression of *Cdh1*, *Acta2*, and *Snail* (Figure 5D). DCB-230 exposure significantly increased expression of all EMT markers tested, including *Cdh1* (1.2 $\pm$ 0.1-fold), *Acta2* (6.9 $\pm$ 1.7-fold), and *Snail* (7.1 $\pm$ 1.7-fold).

EMT Contributes to Airway Smooth Muscle Remodeling Observed after Exposure to DCB-230

We have previously shown that inhalation exposure to DCB-230 at environmentally relevant concentrations in neonatal rats results in persistent pulmonary dysfunction, including airway hyperresponsiveness (AHR) to methacholine, which is a hallmark of asthma. Changes in pulmonary function were associated with distinct changes in pulmonary architecture, including septal destruction, subepithelial fibrosis, and an increase in smooth muscle mass in the peribronchial regions (9). We hypothesized that the persistence in airway dysfunction was due to airway smooth muscle remodeling and tested this hypothesis in neonatal rats exposed to DCB-230 for 20 min/d for 7 days at 200 μ g/m³. Lung sections from exposed rats were stained to visualize epithelial and airway smooth muscle cells and compared with age-matched sections from neonates exposed to filtered air or a nonradical particle control (DCB-50) (Figures E2A and E2B). Remarkable changes in airway architecture were observed after 7 days of exposure. These changes included irregular distribution of epithelial cells and increased smooth muscle mass in the peribronchial regions. Morphometric analysis revealed significant differences in the thickness of α -SMA (Figure E2C) and collagen I (Figure E2D).

To investigate EMT *in vivo*, we moved our studies to a mouse model. Neonatal mice were exposed to DCB-230 as described

above, and histological, immunohistochemical, and gene expression markers associated with EMT were evaluated. As in the neonatal rats, DCB-230 exposure increased peribronchial smooth muscle mass (Figure 6A). Immunohistochemical staining for E-cad and α -SMA revealed cells in the subepithelial layer with distinct E-cad staining, which appear to have migrated from the epithelial layer into the smooth muscle layer (Figure 6B, arrowhead). In addition, cells showed faint expression of E-cad and α -SMA expression (yellow with DAPI staining nuclei) at 4 days after exposure (Figure 6B). Although no coexpressing cells were observed at 7 days after exposure, a substantial increase in α -SMA was evident in the subepithelial layer, analogous to that observed in the lungs of neonatal rats.

Snail expression was probed in the whole lung (Figure 6C) and airway epithelial preparations (Figure 6D). *Snail* expression was elevated in the whole lung at 4 days after exposure (2.6 $\pm$ 0.1-fold) but returned to homeostatic expression levels by 7 days after exposure. *Snail* expression in airway epithelial cells were slightly elevated at 4 days after exposure (1.3 $\pm$ 0.4-fold) and significantly elevated compared with air-matched epithelial cells at 7 days after exposure (1.5 $\pm$ 0.1-fold). *Cdh1* and *Sod2* gene expression were also probed in the whole lung (Figures 6D and 6E). *Cdh1* expression increased slightly at 4 days after exposure but was significantly decreased (0.4 $\pm$ 0.1-fold) at 7 days after exposure, whereas *SOD2* expression was significantly decreased at 4 days after exposure (0.5 $\pm$ 0.03-fold) and not significantly different from controls (0.8 $\pm$ 0.2-fold) at 7 days after exposure. Consistent with oxidative stress observed after *in vitro* exposure, the GSH/GSSG ratio in bronchoalveolar lavage fluid was significantly decreased in DCB-230–exposed mice (Table 1) at 4 and 7 days after exposure.

Lineage tracing experiments were performed using the CCSP-iCre mice and ROSA reporter mice to verify that cells entering the smooth muscle layer were at least in part epithelial

Figure 6. Evidence of EMT in the lung of neonatal mice exposed to DCB-230. (A) Hematoxylin and eosin staining of lung sections from vehicle- or DCB-230-exposed neonatal mice at 4 and 7 days after exposure. Note the epithelial irregularities and increased smooth muscle mass (arrows). (B) Alterations in bronchiolar epithelial structure were verified by staining epithelial cells with E-cad (green) and airway smooth muscle cells with α -SMA (red) and by counterstaining nuclei with DAPI. Coexpression of E-cad and α -SMA expression was observed at 4 days after exposure (arrowheads) but not at 7 days after exposure. (C) *Snai1* expression increased in the whole lung and airway epithelial preparations from neonatal mice exposed to vehicle or DCB-230. Sample expression was normalized to *Hprt1* and plotted as mean fold change $\pm$ SEM compared with time-matched vehicle controls. (D) *Cdh1* (E-cad) expression was

not statistically different in the whole lung of DCB-230-exposed mice at 4 days after exposure, but *Cdh1* was significantly decreased by 7 days after exposure. (E) Conversely, *Sod2* expression was significantly decreased at 4 days after exposure and returned to near baseline levels at 7 days after exposure (* $P < 0.05$; $n = 4$).

derived. IHC for β -galactosidase (β -gal) from airway epithelial cells and α -SMA from mesenchymal cell fate was performed. Age-matched reporter mice exposed to vehicle were included as controls (Figure 7). In neonates exposed to DCB-230 for 7 days, cells staining positive for β -gal and α -SMA were present beneath stretches of airways where disorganized epithelial formations could be observed (Figure 7). These data suggest that epithelial cells undergo partial dedifferentiation associated with dynamic changes in E-cad expression and acquire a mesenchymal phenotype associated with an increase in α -SMA expression. In support of this, we observed an increase in the number of α -SMA-positive cells in the airways at 7 days after exposure in both our neonatal rat (Figure E2) and mouse models (Figure E3). Colocalization studies on airway images from vehicle or DCB-230-treated mice are shown in Figures E4 and E5, respectively.

DISCUSSION

Asthma incidence in urban areas has been steadily increasing (29). Several studies have established relationships between PM exposure and asthma incidence and exacerbation (4, 30). Although the mechanisms of this interaction are not well understood, PM is thought to exert adjuvant immune effects by inducing proinflammatory responses (eosinophilic/neutrophilic inflammation and T cell recruitment), which further exacerbate underlying lung pathologies (31). Recently, the involvement of EMT has garnered more attention as a parallel component of asthma pathology (32–35). These studies suggest a strong relationship between asthmatic airway remodeling and EMT (36) and suggest that exposures to the developing lungs (i.e., lungs of the neonatal, infant, and young child) may have more devastating

consequences (37). We therefore tested whether exposure to PM containing an EPFR (i.e., DCB-230) causes EMT and whether these changes affect developing lung architecture.

From our previous studies with EPFR-containing particles, it was unclear that DCB-230 was taken into cells (14, 15, 24). Here, we observed uptake and absorbance of DCB-230 onto BEAS-2B bronchiolar epithelial cells using TEM. We also observed uptake in several other cell types, including macrophages and fibroblasts (data not shown). In agreement with our previous studies, we found substantial oxidative stress (glutathione depletion, increased GSSG/GSH ratios, and increased 8-IP levels) in cells exposed to EPFR-containing particles (14, 15, 24). In this study, oxidative stress was observed at a substantially lower dose (20 $\mu\text{g}/\text{cm}^2$). This led us to investigate the intracellular location of particles and cellular compartments receiving the oxidative burden. In agreement with several studies (38, 39), we observed changes in Rab5a⁺ endosome (data not shown) as well as lysosome morphology and location within exposed cells,

TABLE 1. REDUCED GLUTATHIONE-TO-OXIDIZED GLUTATHIONE RATIOS IN THE BRONCHOALVEOLAR LAVAGE FLUID WERE SIGNIFICANTLY DECREASED AT 4 AND 7 DAYS AFTER 1,2-DICHLOROBENZENE EXPOSURE

Exposure	Day 4	Day 7
Vehicle	9.24 $\pm$ 2.18	11.42 $\pm$ 1.31
DCB-230	2.41 $\pm$ 0.42*	4.12 $\pm$ 0.31*

Definition of abbreviation: DCB-230 = 1,2-dichlorobenzene.

All measurements were made 24 h after exposure ($n = 5$ animals per group). Data are means $\pm$ SEM.

* $P < 0.05$.

Figure 7. Epithelial cell fate mapping after DCB-230 exposure. The fate of airway epithelial cells from vehicle-exposed (*left column*) or DCB-230-exposed (*right column*) mice was confirmed using CCSP-iCre reporter mice crossed with R26ROSA.LacZ mice. β -galactosidase (β -gal) produced by epithelial cells was stained with a β -gal antibody (*green*), and airway smooth muscle cells were labeled with α -SMA antibody (*red*). Panels from top to bottom: β -gal and DAPI; α -SMA and DAPI; β -gal and α -SMA; and β -gal, α -SMA, and coexpression (*magenta*) as derived from the Zeiss AxioVision colocalization module and imaged on a Zeiss Axio Observer Z1 inverted microscope. Air-exposed reporter mice served as age-matched controls. In mice exposed to DCB-230, cells coexpressed β -gal and α -SMA at 7 days after exposure. Representative sections from vehicle and DCB-230-exposed mice are shown ($n = 8$). Scale bar, 20 μ m.

indirectly supporting the endosome/lysosome compartment as the temporary DCB-230 destination after uptake. Relocation of these compartments to the perinuclear region has been observed after PM uptake (40) and usually accompanies loss of focal adhesions (41). DCB-230 mimicked these responses. Shortly after exposure, lysosomes trafficked toward the nucleus, increased in size, and decreased in number with time. This decrease in lysosomes was immediately preceded by the evidence of lipid peroxidation in the cytoplasm and perinuclear region.

Hydroxyl radical and superoxide anion have been implicated as principal agents that lead to lipid peroxidation (42). Our recent characterizations of DCB-230 indicate that, in physiological solutions, phenoxy- and semiquinone-type radicals are stabilized

on the particle surface (15). These radicals participate in redox cycles involving Fenton chemistry (14), leading to the generation of hydroxyl radicals and causing increases in antioxidant responses. In fact, the cellular response to EPFR-containing particles has been linked to increased production of catalase (15) and here to increased SOD2 production. As suggested in previous studies, oxidative stress is likely the factor that determines the biological activity due to PM exposure (6). Downstream of decreased GSH/GSSG ratios, intracellular lipid peroxidation in the perinuclear region was observed and coincided with altered cell morphology. This suggests that the production of radicals by EPFRs is likely contributing to cytotoxicity and possibly EMT at environmentally relevant doses.

Although several studies have identified characteristics of EMT in exposed cells, such as extracellular signal-regulated kinase phosphorylation, morphological changes, and loss of epithelial junctions (13, 43), EMT due to PM exposure are not prevalent in the literature. However, several EMT triggers are activated by oxidative stress, including SNAIL, p38, and hypoxia-inducible factor (44–47). Additionally, PM-mediated oxidative stress has been shown to modulate proteins involved in focal adhesions and actin dynamics (46, 48, 49). We have previously identified cofilin-1 as highly up-regulated in DCB-230-exposed neonatal lungs wherein significant airway remodeling was also observed; therefore, we studied actin dynamics in epithelial cells after DCB-230 exposure. Decreased focal adhesions in exposed epithelial cells, increased mesenchymal morphology, and increased actin polymerization led us to consider EMT as an epithelial cell response after DCB-230 exposure. With the use of an EMT array, we identified increased expression of E-Cad repressors *SNAIL*, *SNAIL2*, *SNAIL3*, *TCF3*, *TCF4*, *ZEB1*, and *ZEB2* along with decreased expression of intracellular junction proteins *CTNGB1*, *DSP*, *NOTCH1*, and *OCN*, all of which support an EMT (50, 51). Additionally, we observed up-regulation of *MMP3*, *MMP9*, *TGFB1*, *TGFB2*, and *TGFB3*, which supports the potential for extracellular matrix breakdown and remodeling. These changes correlated with changes in the expression of pFAK, collagen I, E-Cad, and α -SMA in DCB-230-exposed epithelial cells and were accompanied by an increase in *SNAIL* expression. These results were corroborated in primary neonatal ALI cultures. Several studies have linked increased SNAIL expression to an EMT phenotype in epithelial cells (19, 50).

The importance of TGF- β in bronchiolar EMT has been shown in recent studies (20, 24, 52). However, monitoring TGF- β in BEAS-2B or neonatal ALI culture supernatants or *in vivo* at 4 or 7 days after exposure revealed no significant change in TGF- β production before or during EMT (data not shown). Furthermore, exposure of BEAS-2B cells to DCB-230 in the presence of TGF- β -neutralizing antibody did not decrease α -SMA protein (data not shown). Finally, the timing of EMT induction by DCB-230 was markedly different compared with that of TGF- β (24 h versus 6 d). Cumulatively, our data suggest that TGF- β is not responsible for the EMT observed after DCB-230 exposure.

Although the precise mechanism responsible for initiating EMT is unclear, oxidative stress induced by DCB-230 may be fueling EMT. PM cytotoxicity has been linked to mitochondrial oxidative stress by several groups. In response to mitochondrial oxidative stress, cells have been shown to up-regulate hypoxia-inducible factor (HIF) α and thus SOD2 (a HIF α responsive gene) to detoxify ROS. Therefore, we shifted our focus to the role of oxidative stress in the cellular response by supplementing SOD2 to exposed cells to boost antioxidant capacity. In support of the pathway presented by Zhou and colleagues (45) and Felton and colleagues (44), we found that supplementing SOD2, either with a cell-permeable or cell-impermeable SOD, was able to reduce

ACTA2 (α -SMA) and *SNAI1* expression while increasing *CDH1* (E-Cad) expression after exposure. The effect was more pronounced with the cell-impermeable SOD, suggesting a potential SOD2 interaction with DCB-230 before uptake, leading to radical neutralization.

Alternatively, given our data and recent literature demonstrating a role for ROS in *SNAI1* induction (53), it is possible that DCB-230-mediated oxidative stress accompanied by lysosomal stress triggers *SNAI1* activation. *SNAI1* activation coupled with a continuous source of radical species (or oxidative stress) may then drive expression of EMT genes and proteins. In total, our data suggest that oxidative stress produced by DCB-230 uptake may overwhelm antioxidant defenses, specifically SOD2 leading to EMT, and explain why several EMT characteristics are present in exposed epithelial cells in the absence of measurable increases in TGF- β .

BEAS-2B cells were used in a majority of the *in vitro* studies because they are derived from the bronchial epithelium (i.e., the site of *in vivo* injury) and retain the ability to undergo EMT differentiation. These cells were obtained at autopsy from noncancerous adults, thus facilitating the need for us to verify our results with this cell line with primary neonatal epithelial cells cultured at ALI. Our data demonstrated that DCB-230 exposure of neonatal epithelial cells also led to morphological changes and protein and mRNA expression changes consistent with EMT. In particular, we observed colocalization of E-cad and α -SMA at sites of DCB-230 injury and increased *Snail* and *Acta2* expression, all of which was in agreement with our BEAS-2B cell line data. We observed a slight, but nonsignificant, increase in E-cad expression in neonatal epithelial cells exposed to DCB-230, which was also observed *in vivo* at 4 days after exposure. This unexplained increase was transient because at 7 days after exposure E-cad expression was markedly and significantly decreased *in vivo*.

Epithelial lineage tracing, using reporter systems driven by airway-specific genes such as CCSP, provide a tool to document EMT *in vivo*. CCSP-iCRE mice produce Cre-recombinase upon expression of CCSP and, when combined with reporter mice, provide a means to trace epithelial cell movement. Our data demonstrate that daily acute exposures to DCB-230 in neonates causes epithelial disruption, decreased GSH/GSSG ratio, and increased expression of markers associated with EMT (i.e., pFAK, *Snail*, etc.) within 3 days of continuous exposure at an environmentally relevant exposure dose. During this period, cells derived from the epithelium could be found in the smooth muscle layer and stain positive for epithelial-derived β -gal and the mesenchymal marker α -SMA. Our findings cumulatively support EMT as a potential process by which EPFR-containing PM induces smooth muscle remodeling, a significant component of the asthma phenotype. Studies that have followed the route of PM deposition after inhalation have readily identified particles in the lung interstitial space within the first few hours of exposure (54, 55). Therefore, these processes may be occurring very shortly after inhalation, and repeated exposure may contribute to progressive airway remodeling. Inhalation of DCB-230 by neonatal rats and mice induced significant changes in the epithelium within 3 days, which persisted for at least 2 m after exposure ceased.

These appreciable alterations in neonatal pulmonary morphology are specifically concerning given the critical stage of lung development. The saccular structure of the neonatal lung allows limited gas-exchange capabilities. Development into a mature lung with a large internal surface area capable of highly efficient gas exchange requires thinning of the alveolar walls, extensive subdivision of saccular lung into alveoli, and growth of the pulmonary capillary network. Exposure to EPFR-containing PM (such as DCB-230) appears to oppose and substantially exacerbate these developmental processes. Research has shown that

increased oxidative stress due to mechanical ventilation in the pre-term human lung results in extensive alveolar fibroproliferation, smooth muscle hyperplasia, and inhibition of distal lung formation leading to long-term pulmonary dysfunction persisting into adulthood. Using this neonatal exposure model, we have shown the potential of EPFR-containing PM to change airway structure during an acute exposure in a neonatal lung. It is of significant concern that these changes in airway structure and function are potentially irreversible. Further studies are needed to address the downstream reversibility of these changes upon later exposure to PM, antigen, or pathogens. In summary, the model EPFR-containing PM, DCB-230, shows potential to substantially influence pulmonary development through induction of EMT, which may be important in determining predisposition to asthma.

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