A Protein Kinase C δ -Dependent Protein Kinase D Pathway Modulates ERK1/2 and JNK1/2 Phosphorylation and Bim-Associated Apoptosis by Asbestos

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Inhalation of asbestos and oxidant-generating pollutants causes injury and compensatory proliferation of lung epithelium, but the signaling mechanisms that lead to these responses are unclear. We hypothesized that a protein kinase (PK)C δ -dependent PKD pathway was able to regulate downstream mitogen-activated protein kinases, affecting pro- and anti-apoptotic responses to asbestos. Elevated levels of phosphorylated PKD (p-PKD) were observed in distal bronchiolar epithelial cells of mice inhaling asbestos. In contrast, PKC δ -/- mice showed significantly lower levels of p-PKD in lung homogenates and *in situ* after asbestos inhalation. In a murine lung epithelial cell line, asbestos caused significant increases in the phosphorylation of PKC δ -dependent PKD, ERK1/2, and JNK1/2/c-Jun that occurred with decreases in the BH3-only pro-apoptotic protein, Bim. Silencing of PKC δ , PKD, and use of small molecule inhibitors linked the ERK1/2 pathway to the prevention of Bim-associated apoptosis as well as the JNK1/2/c-Jun pathway to the induction of apoptosis. Our studies are the first to show that asbestos induces PKD phosphorylation in lung epithelial cells both *in vivo* and *in vitro*. PKC δ -dependent PKD phosphorylation by asbestos is causally linked to a cellular pathway that involves the phosphorylation of both ERK1/2 and JNK1/2, which play opposing roles in the apoptotic response induced by asbestos. (Am J Pathol 2009, 174:449–459; DOI: 10.2353/ajpath.2009.080180)

Asbestos is a group of naturally occurring mineral fibers that are linked to the development of lung cancer, me-

sothelioma, and pleural and pulmonary fibrosis, ie, asbestosis.^{1,2} The mechanisms leading to asbestos-related diseases are still unclear, but oxidative stress due to phagocytosis of longer fibers, iron-driven generation of oxidants from fiber surfaces, and depletion of cellular antioxidants are linked to cell injury and inflammation.^{3–6}

Bronchiolar and alveolar type II epithelial cells, which first encounter asbestos fibers after inhalation, are key cell types in asbestos-associated inflammation and fibroproliferation.² Initial cell reactions to asbestos include epithelial cell injury, ie, apoptosis and necrosis,^{5,6} which may lead to compensatory cell proliferation^{7,8} and the production of inflammatory and fibrogenic cytokines.^{8–10} Asbestos-induced signaling mechanisms governing these cell responses appear to involve a broad variety of cascades including the mitogen-activated protein kinases (MAPK),^{3,7,11,12} nuclear factor- κ B (NF- κ B),^{9,13,14} and the protein kinase (PK)C^{10,12,15,16} and A families.¹⁷

A critical signaling protein involved in asbestos signaling is PKC δ , which is known to be activated in bronchiolar and alveolar epithelial cells *in vivo* and *in vitro*^{10,12,16} via increased formation of diacylglycerol.¹⁸ We have shown that PKC δ governs apoptosis via an oxidant-dependent mitochondrial pathway after exposure of lung epithelial cells to asbestos fibers.¹⁶ Recent studies comparing PKC δ +/+ and PKC δ -/- mice also reveal an important role of PKC δ in metalloproteinase expression as well as cytokine production *in vitro* and *in vivo*.^{10,15} A variety of other studies also link PKC δ to either pro-apoptotic or anti-apoptotic events depending on the stimulus and cell type.^{19,20}

In this study, we focused on PKD as a potential link between PKC δ , activation of MAPKs and downstream repercussions such as expression of *fos/jun* proto-onco-

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genes and apoptosis in asbestos-exposed lung epithelium. PKD is a serine/threonine protein kinase classified as a subfamily of the Ca^{2+} /calmodulin-dependent kinase superfamily.²¹ PKD1, which includes mouse PKD and its human homolog PKC μ , is the most extensively studied PKD.²² The other two members of this family include PKD2²³ and PKD3, (originally PKC ν).²⁴ Conserved regions of PKDs include a phosphorylation-dependent catalytic domain, a pleckstrin-homology domain that inhibits the catalytic activity, and cysteine-rich motifs that recruit PKD to the plasma membrane. PKC δ is proposed to interact with the pleckstrin-homology domain of PKD, transphosphorylating its activation loop at Ser744 and Ser748, and leading to PKD activation.²⁵ In addition, PKD can be activated through the Src-Abl pathway by tyrosine phosphorylation of Tyr463 (T463) in the pleckstrin-homology domain after oxidative stress,²⁶ as well as by caspase-mediated proteolytic cleavage²⁷ and by bone morphogenetic protein 2.²⁸ Downstream targets of PKD signaling include several important signaling molecules such as ERK1/2, JNK1/2, and NF- κ B,^{21,26,29,30} but how these affect functional ramifications of carcinogens, such as asbestos, are unclear.

The BH3-only protein, Bim, is a pro-apoptotic member of the Bcl-2 family that links stress-induced signals to the core apoptotic machinery.^{31,32} There are three different splice variants of the Bim gene encoding short, long, and extra-long Bim proteins (BimS, BimL, and BimEL).³³ BimS-induced apoptosis requires mitochondrial localization but not interaction with anti-apoptosis proteins,³⁴ whereas BimL is bound to microtubules and is less cytotoxic.³⁵ Disruption of BimL binding to microtubules via JNK-dependent phosphorylation can cause its redistribution to the mitochondria and induction of pro-apoptotic machinery.³⁶ BimEL is post-translationally regulated by ERK1/2, which promotes its phosphorylation and rapid dissociation from Mcl-1 and Bcl-x(L)³⁷ and proteasomal degradation.³⁸

We reveal here that PKD is involved in multiple signaling events after asbestos inhalation and *in vitro*. Specifically, PKD is a downstream effector of PKC δ and modulates phosphorylation of both ERK1/2 and JNK1/2 in lung epithelial cells after asbestos exposure. Our data also suggest that PKD inhibits apoptosis through an ERK1/2-mediated destabilization of the pro-apoptotic BH3-only protein, BimEL. The fact that PKD is an important signaling molecule in MAPK signaling and survival after cell injury by asbestos may have important therapeutic implications in asbestos-related diseases.

Materials and Methods

Cell Cultures and Inhibitors

A contact inhibited, non-transformed murine alveolar type II epithelial cell line (C10),³⁹ was grown in CMRL 1066 medium supplemented with L-glutamine (2 mmol/L), penicillin/streptomycin (100 μ g/ml), and 10% fetal bovine serum (GIBCO BRL, Rockville, MD). Cells were grown to near confluence; then complete medium was replaced

with serum-free (maintenance) medium for 16 hours before exposure to asbestos or the phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (100 ng/ml; Calbiochem, La Jolla, CA). Crocidolite asbestos fibers [$\text{Na}_2(\text{Fe}^{3+})_2(\text{Fe}^{2+})_3(\text{OH})_2(\text{Si}_8\text{O}_{22})$], National Institute of Environmental Health Sciences reference sample) were suspended in Hanks' balanced salt solution (GIBCO BRL, Rockville, MD) at a concentration of 1 mg/ml, sonicated, triturated 10X through a 22-gauge needle to obtain a homogenous suspension, and added directly to medium at a final concentration of 5 μ g/cm² area culture dish for the times indicated. This concentration was selected because it causes apoptosis in C10 cells at 24 hours followed by compensatory proliferation at 72 hours.⁷

In some experiments, the NF- κ B inhibitor, Bay 11-7082 (5 and 10 μ mol/L; Sigma, St. Louis, MO), the JNK inhibitor SP600125 (10 and 20 μ mol/L; Calbiochem, La Jolla, CA), or the ERK1/2 inhibitor U0126 (10 μ mol/L; Sigma, St. Louis, MO) were added to C10 cells 1 hour before asbestos at effective concentrations of inhibitors reported in the literature.^{40–42} Control cultures received medium without agents and were treated identically. All experiments were performed in triplicate or more.

Inhalation Experiments

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals following protocols approved by the University of Vermont Institutional Animal Care and Use Committee. PKC δ –/– mice bred into the C57Bl/6 background were characterized as PKC δ +/+ or PKC δ –/– as described previously.^{10,43} In brief, mice (8 to 12 weeks of age) were exposed to ambient air or the National Institute of Environmental Health Sciences reference sample of chrysotile asbestos (7 mg/m³ air, 6 hours/day, 5 days/week) for 9 or 40 days as described previously.^{9,10,12} The mice were euthanized using an intraperitoneal injection of pentobarbital (Abbott Laboratories, Abbott Park, IL), the lungs instilled with PBS, and lobes tied off using sutures. The left lung lobe was immersed in optimal cutting temperature embedding compound (Tissue-Tek, Torrance, CA) before snap-freezing in liquid-cooled isopentane and storage at –80°C until sectioning. Other lobes were used for Western blot analyses.

Transient Transfections with Small Interfering RNA

The siControl Non-Targeting small interfering (si)RNA #2 (scrambled control), Smartpool mouse PKD, or PKC δ siRNA (50 nmol/L, Dharmacon, Lafayette, CO) were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. The efficiency of PKD and PKC δ knockdown was determined by Western blot analysis after 48 and 72 hours.

Immunofluorescence Techniques

To determine whether phosphorylated PKD was expressed in lung after inhalation of asbestos, 10 μ m thick sections of lung tissue were fixed in 4% paraformaldehyde for 7 minutes, washed in PBS, and permeabilized with methanol for 10 minutes at -20°C . After washing in PBS, sections were treated for antigen retrieval in 1% SDS for 5 minutes, washed with PBS, and then incubated with a blocking solution containing 10% normal goat serum in 2% bovine serum albumin (BSA)/0.1% Triton X-100/PBS solution for 1 hour at room temperature. After aspiration of blocking solution, primary antibody (rabbit polyclonal phospho-Ser744/Ser748 PKD, Cell Signaling Technology, Danvers, MA; 1:1000) diluted in 2% BSA plus 0.1% Triton X-100 in PBS (BSA/PBS-T) was added, and sections were incubated overnight at 4°C . Sections were then washed with PBS, and incubated with secondary antibody (AlexaFluor 568 goat-anti-rabbit IgG, Molecular Probes, Carlsbad, CA; 1:400 in PBS) for 1 hour at room temperature. Controls consisted of sections incubated with secondary antibody alone. Sections then were washed in PBS followed by incubation with the nuclear counterstain, YOYO-1 iodide (Molecular Probes; 1:10000), 1 unit/ml RNase, and 0.1% sodium azide in BSA/PBS-T for 30 minutes at room temperature. After washing in PBS, coverslips were mounted onto slides using AquaPolyMount (Polysciences, Inc. Warrington, PA). For each section, confocal images were collected in fluorescence modes using a Bio-Rad MRC1024ES confocal scanning laser microscope (Bio-Rad, Hercules, CA).

To determine whether PKD-driven signaling pathways occurred in the same cell, confluent C10 cells were exposed to H_2O_2 , a mediator of asbestos-induced lung injury^{4,44} at concentrations (0.5 and 5.0 mmol/L for 30 minutes) sufficient to activate JNK1/2 and induce cell death.⁴⁵ C10 cells were grown to confluency in 4 well-chambered slides (BD Biosciences, San Jose, CA), and untreated and H_2O_2 -exposed cells were fixed for 20 minutes in 100% methanol at -20°C . After a wash in PBS and blocking for 1 hour in 10% goat serum (Jackson ImmunoResearch, West Grove, PA) and 3.7% mouse on mouse IgG blocking reagent (Vector Labs, Burlingame, CA) in PBS, cells were incubated overnight at 4°C with primary antibodies [mouse anti-pERK1/2 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and rabbit anti-pJNK1/2 (Cell Signaling Technologies, Danvers, MA)] at 1:50 dilutions in 1% BSA in PBS. Controls were incubated with 1% BSA in PBS alone. Cells were washed in PBS before incubation with the secondary antibodies, Alexa Fluor 568 goat anti-mouse IgG and Alexa Fluor 647 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA), that were diluted 1:400 in PBS at room temperature for 1 hour in the dark. After washing in PBS, a 1:20,000 dilution of Sytox Green (Invitrogen, Carlsbad, CA) was added for 10 minutes at room temperature in the dark. Cells were then washed in distilled water, allowed to air dry, coverslips mounted with Aqua Poly/Mount (Polysciences Inc., Warrington, PA), and stored at 4°C until examination using a Bio-Rad

MRC1024ES confocal scanning laser microscope (Bio-Rad, Hercules, CA).

Immunoprecipitation Experiments

To test the hypothesis that physical interactions between: 1) PKC δ , PKD, and ERK1/2 proteins, and 2) PKD, JNK1/2, and ERK1/2 occurred, C10 cells grown in 100-mm culture dishes were washed three times with ice-cold PBS and collected in lysis buffer (20 mmol/L Tris pH 7.6, 1% Triton X-100, 137 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L Na SO_4 , 10 mmol/L NaF, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin) on ice for 30 minutes. Cells were then sonicated and centrifuged at $14,000 \times g$ for 15 minutes at 4°C . Supernatants were collected, and protein concentrations were determined using the Bradford assay (Bio-Rad, Richmond, CA). We first used 300 μg of protein that was immunoprecipitated for 2 hours at 4°C with PKD antibody (1:100, Cell Signaling), and the antigen-antibody complexes collected by incubation with Agarose protein A (Life Technologies, Inc.) for 1 hour at 4°C . After pellets were washed three times with lysis buffer, remaining immunocomplexes were collected in SDS sample buffer. After boiling the immunocomplexes for 5 minutes at 95°C , Western blots with antibodies to ERK1/2, PKC δ , and PKD were then performed (see below). We then used an identical procedure to immunoprecipitate protein with a JNK1/2 antibody (Cell Signaling) and probed Western blots with antibodies to ERK1/2 and PKD.

Western Blot Analyses

Cells were exposed to agents as described above, the medium aspirated, and cells washed twice with ice-cold PBS prior collection in 4X sample buffer (200 $\mu\text{mol}/\text{L}$ Tris, pH 6.8, 4% SDS, 4 mg/ml β -mercaptoethanol, 40% glycerol, 2 $\mu\text{mol}/\text{L}$ pyronin-Y). The amount of protein was determined using the RC DC protein assay (Bio-Rad, Hercules, CA). A total of 30–60 μg protein was separated by 10% SDS-PAGE and transferred to nitrocellulose. Western blots were performed as described previously,⁷ using antibodies specific to total and phosphorylated PKD (rabbit polyclonal anti-PKD, 1:1000, rabbit polyclonal anti-phospho-Ser 744/748 PKD 1:1000, Cell Signaling Technology, Danvers, MA), total and phosphorylated ERK1/2 (rabbit polyclonal anti-ERK1/2, 1:1000; rabbit polyclonal phospho-ERK1/2 1:500, and mouse monoclonal anti-ERKp42 1:1000; Cell Signaling Technology, Danvers, MA), total and phosphorylated JNK1/2 (rabbit polyclonal JNK1/2, 1:200, rabbit polyclonal phospho JNK1/2, 1:1000, Cell Signaling Technology, Danvers, MA), total and phosphorylated c-Jun (rabbit polyclonal c-Jun at 1:1000, rabbit polyclonal phospho c-Jun at 1:500, Cell Signaling Technology, Danvers, MA), total PKC δ (rabbit polyclonal PKC δ at 1:1000, Santa Cruz, Santa Cruz, CA), total β -Actin 1:2000 (Abcam, Cambridge, MA), and total Bim (rabbit polyclonal anti-Bim at 1:500, Cell Signaling Technology, Danvers, MA). Quanti-

Figure 1. PKD activation in bronchiolar epithelial cells occurs after inhalation of chrysotile asbestos by wild-type (PKC δ ^{+/+}), but not PKC δ knockout (PKC δ ^{-/-}) mice. An antibody recognizing the PKD activation loop (anti-pSer 744/748) was used to detect p-PKD (red; **arrow**), and nuclei (green) were stained with YOYO-1 iodide before examination of lung sections ($N = 4$ mice/group/time point) using a confocal scanning laser microscope. Note red fluorescence predominantly in bronchiolar epithelial cells lining the bronchiolar lumen (BL) in wild-type (PKC δ ^{+/+}) mice (**B** and **C**) exposed to asbestos, which was decreased in PKC δ ^{-/-} mice (**E** and **F**). (**A**) and (**D**) depict lungs from sham, clean air exposed mice. All micrographs at original magnification $\times 400$; scale bar = 50 μ m.

tyOne was used to quantify band density, and values were normalized to respective total kinase. Blots are representative of three different experiments. In some experiments, TPA was added for 30 minutes as a positive control for phosphorylation of PKC δ or PKD.

Detection and Quantitation of Apoptosis

To determine whether modulation of PKD levels altered cell death in lung epithelial cells, detection of apoptosis was performed as previously described.⁷ Briefly, C10 cells grown on glass coverslips were fixed in methanol for 24 hours at -20°C , boiled for 5 minutes in PBS containing 5 mmol/L MgCl₂, then immersed in ice-cold PBS for 10 minutes followed by blocking with 40% fetal bovine serum for 15 minutes. Cells were then incubated with Apos-tain F7-26 (10 $\mu\text{g/ml}$; Alexis Biochemicals, San Diego, CA) for 30 minutes at room temperature, followed by peroxidase-conjugated secondary antibody (goat anti-mouse IgM, 15 $\mu\text{g/ml}$; Jackson Laboratories, West Grove, PA) for 30 minutes at room temperature. To visualize secondary antibody binding, the peroxidase substrate diaminobenzidine (DakoCytomation, Carpinteria, CA) was used. Nuclei were counterstained with hematoxylin, and coverslips were mounted onto slides with Aqua-PolyMount (Polysciences, Warrington, PA) for subsequent examination using bright field light microscopy. To determine the numbers of apoptotic cells and total cell numbers per field, five random fields were evaluated at original magnification $\times 400$, on each coverslip.

Statistical Analysis

Results were evaluated by one-way analysis of variance using the Student-Newman-Keul's procedure for adjustment of multiple comparisons. Differences with P values ≤ 0.05 were considered statistically significant. Data from *in vivo* experiments were evaluated by the Student's t -test using paired comparisons.

Results

Asbestos-Mediated PKD Activation is PKC δ -Dependent *In Vivo* and *In Vitro*

We first used immunostaining and Western blot analyses to determine whether increased amounts of phosphorylated PKD in lung were observed after inhalation of asbestos. In comparison with wild-type mice (PKC δ ^{+/+}) inhaling clean air (shams; Figure 1A), PKC δ ^{+/+} mice inhaling asbestos showed increased immunostaining for phosphorylated PKD (p-PKD) that was more extensive after exposure to asbestos for 9 days (Figure 1B). p-PKD was localized predominantly in the apical cytoplasm of bronchiolar epithelial cells and was still increased after 40 days of exposure to asbestos, where some staining was also observed in alveoli and associated inflammation (Figure 1C). In contrast, sham and asbestos-exposed PKC δ ^{-/-} mice that develop altered inflammation and less fibrogenesis after exposure to chrysotile asbestos^{10,15} did not show increased p-PKD expression (Figure 1, D–F). No fluorescence was observed in the absence of a primary antibody (data not shown). Western blot analyses using lung lysates also exhibited p-PKD in PKC δ ^{+/+} wild-type mice that was reduced in lungs of PKC δ ^{-/-} mice (Figure 2, A and B).

We next used a murine lung epithelial cell line (C10) to further confirm that phosphorylation of PKD was induced by asbestos *in vitro* as suggested by our *in vivo* immunohistochemistry and Western blot experiments. Western blot analyses confirmed that asbestos-induced PKD phosphorylation in epithelial cells peaked from 3 to 10 hours, but levels returned to those observed in control cells at 48 hours (Figure 3, A and B). We also used a siPKC δ construct to determine whether p-PKD levels induced by asbestos would be diminished. In these experiments, TPA-treated cells were used as a positive control for p-PKD. As shown in Figure 3C, transfection of cells with siPKC δ decreased PKC δ . Moreover, as quantitated in Figure 3D, levels of

Figure 2. PKD is activated by asbestos in whole lung homogenates of PKCδ^{+/+} and PKCδ^{-/-} mice as revealed by Western blot analyses. Lungs of sham air exposed (9 days) and mice exposed to chrysotile asbestos for 9 or 40 days were examined by Western blot analysis using antibodies to p-PKD and total PKD as described in Materials and Methods. **A:** Representative Western blots. Blots in (A) were probed with an antibody to β-Actin to verify equal amounts of protein loaded in lanes; **B:** Quantitation of data. Bars = Mean ± SEM.

p-PKD were markedly reduced in C10 cells transfected with siPKCδ in comparison with the scrambled control vector.

PKD Signaling by Asbestos is Upstream of ERK1/2 and JNK1/2 Phosphorylation

To determine whether PKD activation was causally related to phosphorylation of ERK1/2 or JNK1/2, we next transfected C10 lung epithelial cells with a siRNA to inhibit PKD expression (siPKD). Western blot analyses confirmed that total PKD levels were negligible in C10 cells transfected with the siPKD but not with a scrambled RNA control (Figure 4A). Moreover, siPKD-transfected cells exhibited significantly decreased ERK1/2 (Figure 4, A and B) and JNK1/2 phosphorylation (Figure 4, C and D) in response to asbestos.

PKD is Critical to Increased Expression of c-Jun by Asbestos in C10 Lung Epithelial Cells

We have reported previously that mRNA expression of members of the *fos/jun* family is increased in rodent lung epithelial cells exposed to asbestos *in vitro*^{46,47} as well as in lungs of mice after inhalation of asbestos⁴⁸. Use of laser capture microdissection in these studies⁴⁸ showed that increases in *c-jun* occurred in distal bronchiolar epithelial cells, sites of impaction of asbestos fibers after inhalation. Based on these observations and because silencing of PKD in C10 cells resulted in dramatic decreases in phosphorylation of JNK1/2 (see Figure 4, C and D), we next focused on whether PKD was causally linked to phosphorylation of c-Jun. Exposure to asbestos resulted in increases in c-Jun phosphorylation and protein levels that peaked at 24 hours (Figure 5, A and B). However, C10 cells transfected with siPKD showed decreased amounts of c-Jun and c-Jun phosphorylation (Figure 5, C and D).

Figure 3. PKD is phosphorylated in C10 lung epithelial cells via a PKCδ-dependent pathway. Cells were homogenized and Western blot analyses were performed using antibodies to PKCδ, p-PKD and total PKD as described in Materials and Methods. **A and B:** Time course study. Blot in (A) was probed with an antibody to β-Actin to verify equal amounts of protein loaded in lanes. **C and D:** Use of siPKD construct. TPA (100 ng/ml) was used as a positive control for p-PKD. All results are typical of three independent experiments. **P* ≤ 0.05 in comparison with sham (0) group. Bars = Mean ± SEM.

Figure 4. Phosphorylation of ERK1/2 and JNK1/2 is decreased after silencing of PKD in C10 lung epithelial cells. C10 cells were transfected with siPKD or scramble control construct before addition of asbestos for 10 hours. Cells were homogenized and Western blot analyses were performed using antibodies to p-ERK1/2 and total ERK1/2 (**A** and **B**); p-JNK1/2 and total JNK1/2 (**C** and **D**). **A** and **C**: Representative Western blots. **B** and **D**: Quantitation of data, as described in Materials and Methods. Results are typical of three independent experiments. * $P \leq 0.05$ in comparison with sham group (0). † $P \leq 0.05$ in comparison with siControl group. Bars = Mean $\pm$ SEM.

Silencing PKD Increases Asbestos-Induced Apoptosis of Lung Epithelial Cells

Asbestos-induced apoptosis in mesothelial and epithelial cells is causally related to activation and translocation of PKC δ via a mitochondrial pathway.¹⁶ We therefore tested whether inhibition of PKD also modified epithelial cell apoptosis. As shown in Figure 6A using the Apostain technique, addition of asbestos fibers to si control-transfected cells caused focal apoptosis at 24 hours that increased at 48 hours. Numbers of asbestos-induced apoptotic cells were significantly increased in C10 cells transfected with the siPKD construct at 48 hours (Figure 6B). In contrast, inhibition of JNK1/2 with the JNK inhibitor SP600125 resulted in inhibition of asbestos-associated apoptosis that was most striking at 48 hours (Figure 6C). Since activation of the NF- κ B pathway in other cell types is regulated by PKC δ and PKD,²⁶ we also exam-

ined effects of inhibiting the NF- κ B pathway on asbestos-induced apoptosis using the I κ B-inhibitor, Bay11-7082.⁴⁰ Results showed that increased apoptosis by asbestos was unaffected by Bay11-7082 at 5 or 10 μ mol/L (Figure 6D). Since we have previously used ERK1/2 small molecule inhibitors, including U0126, to show that the duration of ERK1/2 signaling by asbestos determines proliferative versus apoptotic responses in C10 cells,^{17,42} experiments using U0126 were not repeated here.

Bim is Decreased after Asbestos Exposure and Increased after Inhibition of PKD or ERK1/2

To examine a possible mechanism to explain the interplay between ERK1/2 and JNK1/2 pathways in the development of asbestos-induced apoptosis, we focused on

Figure 5. Asbestos-induced c-Jun phosphorylation is inhibited in C10 lung epithelial cells transfected with siPKD. Cells were homogenized, and Western blot analyses were performed using antibodies to p-c-Jun, total c-Jun, and β -Actin as described in Materials and Methods. **A**: Western blots of representative time course study. **B**: Quantitation of data. **C**: Representative Western blots after using the siPKD construct or a scrambled control construct (si-control). **D**: Quantitation of assays using siPKD or si-control transfected C10 cells. Results are typical of three independent experiments. * $P \leq 0.05$ in comparison with unexposed cells (0), † $P \leq 0.05$ in comparison with si-control group at the same time. Bars = Mean $\pm$ SEM.

Figure 6. Asbestos-induced apoptosis is increased after silencing of PKD in part through a JNK-dependent, but NF- κ B independent pathway. C10 epithelial cells were transfected with siPKD or a si scramble control before exposure to asbestos, and the Apostain procedure was performed as described in Materials and Methods. **A:** Immunocytochemistry using light microscopy. **Arrow** shows cell staining positively for apoptosis. **B:** Quantitation of data from three independent experiments. **C:** Quantitation of data in studies using the JNK inhibitor SP600125 (SP) at 10 and 20 μ mol/L. **D:** Quantitation of data in studies using the NF- κ B inhibitor, Bay 11-7082 (Bay) 5 μ mol/L and 10 μ mol/L. * $P \leq 0.05$ in comparison with unexposed cells (0), † $P \leq 0.05$ in comparison with sicontrol or solvent (dimethyl sulfoxide) control group at same time point. Bars = Mean $\pm$ SEM.

the pro-apoptotic BH3-only protein Bim that can be stabilized by JNK1/2⁴⁹ and degraded after activation of ERK1/2.^{50,51} Western blots in Figure 7, A and B show that

protein levels of both BimL (16KD) and BimEL (23KD) decreased in C10 cells after asbestos exposure from 10 to 48 hours. No BimS was detected in C10 lung epithelial

Figure 7. BimEL (23kD) and BimL (16kD) proteins are decreased after addition of asbestos to C10 lung epithelial cells (**A**, **B**) and up-regulated after silencing of PKD (**C**) or inhibition of ERK1/2 using U0126 (**D**). **A:** Representative Western blot and (**B**) quantitation of data. **C:** Quantitative data showing that BimEL (23kD) is increased in siPKD transfected cells. **D:** Quantitative data showing that U0126, but not SP600125, prevents asbestos-associated decreases in BimEL. The ERK1/2 inhibitor, U0126 (10 μ mol/L), and JNK1/2 inhibitor, SP600125 (SP; 10 μ mol/L) were added before asbestos as described in Materials and Methods. * $P \leq 0.05$ in comparison with unexposed cells (0); † $P \leq 0.05$ in comparison with siControl at same time point. Bars = Mean $\pm$ SEM.

Figure 8. Immunoprecipitation (IP) experiments showing association of PKCδ, PKD, and ERK1/2 (**A, B**) and PKD, JNK1/2, and ERK1/2 (**C, D**) after immunoprecipitation of protein using antibodies to PKD (**A, B**) or JNK1/2 (**C, D**) respectively, in C10 lung epithelial cells. **A** and **C** show representative Western blots; and **B** and **D** show quantitation of data from duplicate experiments. * $P \leq 0.05$ in comparison with respective groups at 0 time. IgG was used as a loading control in **C** and **D**. IB = Immunoblotting. Bars = Mean $\pm$ SEM.

cells. As shown in Figure 7C, silencing of PKD significantly increased BimEL levels. Pretreatment of cells with the JNK inhibitor, SP600125 (10 μ mol/L), failed to alter asbestos-associated decreases in BimEL protein, whereas inhibition of ERK1/2 using U0126 (10 μ mol/L) increased amounts of BimEL at both 10 and 24 hours post asbestos exposure (Figure 7D). These data suggest that activation of ERK1/2 is in part responsible for the inhibition of Bim-associated apoptosis by asbestos, whereas JNK is pro-apoptotic, presumably by many mechanisms described in the literature.⁵²

PKD Interacts with PKCδ, ERK1/2, and JNK1/2 Proteins in C10 Lung Epithelial Cells

To determine whether PKD physically interacted with PKCδ and MAPK proteins in C10 cells, we performed immunoprecipitation studies using an antibody to PKD (Figure 8, A and B) or JNK1/2 (Figure 8, C and D), as described in Materials and Methods. As shown in Figure 8, A and B, both PKCδ and ERK1/2 associated with PKD protein ($P \leq 0.05$) after exposure to asbestos for 10 and 24 hours. Although similar trends were noted (Figure 8D) after use of an antibody to precipitate JNK1/2, variability between groups precluded statistically significant differences.

p-ERK1/2 and p-JNK1/2 Signaling Events Occur in the Same C10 Lung Epithelial Cell

Data in Figure 8 show interaction of PKD with PKCδ and MAPK proteins. However, it is unclear whether pro- and anti-apoptotic signaling events caused by activation of JNK1/2⁴⁵ and ERK1/2,^{7,12,42} respectively, occur in C10 cells simultaneously in the same cell. Since asbestos-induced phosphorylation of ERK1/2 is either transient or

sustained depending on the cell outcome, ie, proliferation or apoptosis, and difficult to detect *in situ* because of the focal presence of fibers on cells, we used H₂O₂, a soluble mediator of asbestos-induced cell and lung injury,^{4,44} to address this question. As shown in Figure 9A, untreated C10 cells show punctate p-ERK1/2 (red) in the cytoplasm. Cells exposed to 0.5 mmol/L H₂O₂ for 30 minutes

Figure 9. Immunofluorescence experiments showing simultaneous induction of H₂O₂-induced pJNK1/2 and pERK1/2 signaling in C10 lung epithelial cells. (**A**) shows untreated confluent C10 cells. (**B** and **C**) show C10 cells exposed to 0.5 mmol/L and 5 mmol/L H₂O₂ for 30 minutes. (**D**) illustrates the lack of p-JNK1/2 or p-ERK1/2 staining in cells stained with secondary antibody alone. Nuclei are stained with Sytox Green. Red dots indicate p-ERK1/2 phosphorylation. All micrographs at original magnification $\times 400$. Bar = 50 μ m.

exhibit occasional JNK1/2 phosphorylation (blue) that is both cytoplasmic and nuclear (Figure 9B). In contrast, cells exposed to lethal concentrations of H₂O₂ (5 mmol/L) exhibit both cytoplasmic and nuclear translocated pJNK1/2 and pERK1/2 as suggested by Western blot analyses in rat lung epithelial⁴⁹ and mesothelial⁵³ cell homogenates (Figure 9C). Figure 9D shows the lack of p-JNK1/2 and p-ERK1/2 reactivity in cells stained with secondary antibody alone.

Discussion

MAPK signaling via oxidative stress is critical to the development of inflammation, carcinogenesis and fibrogenesis by asbestos or cigarette smoke,³ although asbestos stimulates a number of signaling pathways in target cells (epithelial, fibroblasts, etc) that may be linked cooperatively or antagonistically to disease development or lung repair. In this study we focused on PKD as a key regulatory molecule affecting asbestos-induced MAPK signaling responses. Moreover, we hypothesized that PKD might be linked to activation of MAPK and downstream events such as apoptosis. Here we demonstrate that PKD is an important molecule in asbestos signaling *in vivo* and *in vitro* and is linked to activation of both JNK1/2 and ERK1/2 in asbestos-induced cell injury. By initially using an animal inhalation model, we showed that asbestos exposure activated PKD in lungs of wild-type, but not PKC δ -/- mice. Although others have shown that PKD is activated by PKC δ *in vitro*,²⁶ to our knowledge, ours is the first study confirming this link in a model of lung injury *in vivo*. Based on the observation that p-PKD was expressed at the apical surface of bronchiolar epithelial cells after inhalation of asbestos, as is p-ERK1/2 in our murine model of fibrogenesis,^{11,12} we explored the hypothesis that asbestos activated PKD in murine lung epithelial cells *in vitro*. Although we reported previously that ERK1/2 is a key signaling molecule induced after inhalation of asbestos or its addition to mesothelial and epithelial cells *in vitro*,^{7,11,12} we show here that both ERK1/2 and JNK1/2 are phosphorylated by asbestos and decreased after silencing of PKD.

In support of our observations, a role for PKD activation of individual MAPKs has been indicated in some studies. For example, overexpression of PKD activates p42 ERK1 that is mediated by consecutive activation of Raf and MEK.⁵⁴ Constitutively active PKD suppresses epidermal growth factor-associated c-Jun phosphorylation in human embryonic kidney cells⁵⁵ whereas constitutively active PKD or PKC inhibition in hepatocytes leads to sustained overactivation of JNK1/2 and c-Jun.⁵⁶ These findings rely on data derived from PKD overexpression studies where its physiological significance is unknown. Moreover, *in vitro* phosphorylation studies show that PKC η (another member of the PKC family)-mediated PKD triggers the p42 MAPK cascade while JNK activation is reduced, providing evidence for dual regulation of MAPK.⁵⁷

Silencing approaches used here reveal the biological role of PKD in control of apoptotic cell death via phosphorylation of both ERK1/2 and JNK1/2 pathways that

play opposing roles in this process. Our results also may explain disparate results on the involvement of PKD in anti-apoptotic or pro-apoptotic pathways as reported by others. For example, in a model of mouse skin carcinoma, increased PKD expression is related to cell proliferation.⁵⁸ Likewise, in pancreatic tissue, PKD overexpression leads to strongly enhanced cell growth and up-regulated anti-apoptotic proteins in pancreatic tumor cells.⁵⁹ In mouse fibroblasts, PKD enhances the expression of genes encoding apoptosis-inhibiting proteins (eg, cIAP-2, inhibitor of apoptosis protein 2).⁶⁰ Conversely, other studies show that PKD is cleaved by caspases after its induction by TNF α and chemotherapeutic drugs, thus sensitizing cells to apoptosis-inducing effects of genotoxic agents.⁶¹ Our data using a concentration of asbestos that induces apoptosis followed by compensation proliferation of C10 cells suggest that a dose responsive, JNK-mediated pathway predominates after exposure to toxic amounts of asbestos. In contrast, as supported by reports showing that PKD induction of ERK1/2 is a survival response in tumor cells,^{59,62} ERK1/2-related anti-apoptotic pathways may predominate at lower concentrations of asbestos.

To explore possible downstream targets of MAPK pathways, we next focused on the pro-apoptotic protein Bim that is known to be regulated by ERK1/2. We show here that Bim levels decrease over time in response to asbestos, a phenomenon dependent on ERK1/2 signaling. Bim is known to be pro-apoptotic because of its ability to activate Bax, and a recent report shows that Bim upregulation is critical to anoikis, a Bax-dependent apoptosis triggered by detachment from the extracellular matrix.⁶³

Several reports implicate ERK1/2 in Bim regulation. For example, after serum withdrawal, Bim is increased through rapid protein stabilization and *de novo* synthesis that can be repressed by activation of ERK1/2 in fibroblasts⁵¹ or epithelial cells,⁵⁰ most likely via a destabilizing process involving the proteasome.^{38,64} Inhibition of ERK1/2 activation also increases the amount of BimEL present in the mitochondrial cellular fraction.⁶⁵ The ability of JNK to phosphorylate BimEL is controversial, but has been reported in neuronal cells.⁴⁹ Other studies show a relationship between forkhead transcription factor activation and Bim-mediated apoptosis⁶⁶ suggesting other pathways involved in regulation or stabilization of Bim, and a recent paper reports that sensitivity to Adriamycin correlates with apoptosis and levels of Bim protein in renal cell carcinomas.⁶⁷

Our work here suggests a chain of signaling events that are initiated by asbestos and/or oxidative stress in individual lung epithelial cells and regulate the development of apoptosis or survival. Asbestos fibers initially interact with the plasma membrane to activate PKC δ via increased hydrolysis of inositol phospholipids and diacylglycerol formation.¹⁸ Asbestos also causes translocation of PKC δ to mitochondria as well as mitochondrial generation of ROS.¹⁶ Immunoprecipitation studies here show that PKC δ , PKD, and ERK1/2 physically interact, consistent with prior observations that these proteins occur in mitochondria.^{16,68,69} The accumulation of mitochondrial ROS is known to lead to mitochondrial activation of PKD,⁶⁸ and tyrosine phosphorylation by Src, which

occurs in C10 cells exposed to asbestos,⁷⁰ may allow further activation of PKD by PKC δ . PKC δ -PKD-mediated phosphorylation of ERK1/2 and JNK1/2 may also occur by mitochondrial ROS, resulting in a predominantly apoptotic response at high concentrations of asbestos and cell homeostasis or proliferation at lower concentrations of fibers. These two MAPK pathways might also interact at the level of the Bim promoter, which contains an AP-1 site.⁷¹ Our observations are consistent with the fact that proliferation and apoptosis are dynamic dose-related phenomena in epithelial and mesothelial cells exposed to asbestos.⁷² Taken together, our results demonstrate that PKC δ -dependent PKD functions as a regulatory pathway affecting the downstream outcomes of MAPK activation by asbestos.

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