p53 Mediates Particulate Matter–induced Alveolar Epithelial Cell Mitochondria-regulated Apoptosis

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Rationale: Exposure to particulate matter (PM) causes lung cancer by mechanisms that are unknown, but p53 dysfunction is implicated.

Objective: We determined whether p53 is required for PM-induced apoptosis in both human and rodent alveolar type (AT) 2 cells.

Methods: A well-characterized form of urban PM was used to determine whether it induces mitochondrial dysfunction (mitochondrial membrane potential change [$\Delta\Psi_m$] and caspase-9 activation), p53 protein and mRNA expression, and apoptosis (DNA fragmentation and annexin V staining) *in vitro* using A549 cells and primary isolated human and rat AT2 cells. The role of p53 was assessed using inhibitors of p53-dependent transcription, pifithrin- α , and a genetic approach (overexpressing E6 or dominant negative p53). In mice, the *in vivo* effects of PM causing p53 expression and apoptosis were assessed 72 h after a single PM intratracheal instillation.

Measurements and Main Results: PM-induced apoptosis in A549 cells was characterized by increased p53 mRNA and protein expression, mitochondrial translocation of Bax and p53, a reduction in $\Delta\Psi_m$, and caspase-9 activation, and these effects were blocked by inhibiting p53-dependent transcription. Similar findings were noted in primary isolated human and rat AT2 cells. A549-p^o cells that are incapable of mitochondrial reactive oxygen species production were protected against PM-induced $\Delta\Psi_m$, p53 expression, and apoptosis. In mice, PM induced p53 expression and apoptosis at the bronchoalveolar duct junctions.

Conclusions: These data suggest a novel interaction between p53 and the mitochondria in mediating PM-induced apoptosis that is relevant to the pathogenesis of lung cancer from air pollution.

Keywords: apoptosis; mitochondria; p53; particulate matter; reactive oxygen species

The p53 tumor suppressor is a transcription factor that regulates the expression of genes involved in the regulation of the cell cycle, DNA repair, and apoptosis. The finding that most human cancers have alterations in p53 or one of its regulatory family members, and that p53 knockout animals have an increased incidence of cancer, supports the importance of normal p53 function (1–3). Cells respond to DNA damage by activating p53-regulated genes involved in delaying cell cycle progression to facilitate DNA repair or, if DNA damage is extensive, promoting apoptosis. Exposure to airborne particulate matter (PM) is asso-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Exposure to airborne particulate matter (PM) causes lung cancer by unclear mechanisms; however, evidence implicates that malignant cells arise from deficient programmed cell death (apoptosis) as well as mutations in p53, a critical DNA damage response protein that is a tumor suppressor.

What This Study Adds to the Field

Our findings that p53 mediates PM alveolar epithelial cell apoptosis in both human and rat cells in part by activating the intrinsic (mitochondria) death pathway suggest a novel interaction between p53 and the mitochondria that is relevant to the pathogenesis of lung cancer from air pollution.

ciated with an increased risk of cardiopulmonary diseases, including lung cancer, resulting in an estimated 500,000 excess deaths worldwide each year (4–7). Despite the magnitude of the public health consequences stemming from PM exposure, the precise mechanisms underlying the pathophysiologic effects of PM have not been established. There is evidence showing that exposure of lung epithelial cells and macrophages to PM results in a generation of reactive oxygen species (ROS), DNA damage, reduction in mitochondrial membrane potential change ($\Delta\Psi_m$), activation of caspase-9, mitochondria-regulated apoptosis, and inflammation (8–12). Humans exposed to environmental pollution have increased levels of several markers of genotoxicity, including DNA adducts, chromosome aberrations, sister chromatid exchanges, 8-oxo-dihydro-2'-deoxyguanosine, DNA strand breaks, ras oncogene activation, and p53 expression (5, 6, 13). There is controversy about the source of ROS production that causes apoptosis after PM exposure, but accumulating evidence implicates components in PM, such as transition metals (e.g., iron, vanadium, and others), polycyclic aromatic hydrocarbons, and quinones, as well as the mitochondria of lung target cells (6). However, the molecular mechanisms by which PM induces mitochondrial dysfunction and apoptosis are unclear.

Although p53 modulates apoptosis by complex and incompletely understood mechanisms, one established pathway involves activating the mitochondria-regulated death pathway by increasing gene expression of proapoptotic stimuli (e.g., Bax, Bak, BH3-only molecules, and others) while inhibiting the expression of antiapoptotic Bcl-2 family members (1, 2, 14–16). DNA-damaging agents can also induce a direct mitochondrial effect of p53, in part due to the BH3-like activity of p53, and in part due to the DNA binding domain of p53 interacting with Bcl-xl, promoting Bax/Bak-induced outer mitochondrial membrane permeabilization (1, 2, 17, 18). Recent evidence shows

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that p53-upregulated modulator of apoptosis is essential for coupling the nuclear and cytoplasmic proapoptotic function of p53 (19). A mechanistic link between mitochondrial ROS production and p53-induced apoptosis has been suggested (20, 21). We recently showed that p53 mediates asbestos-induced alveolar epithelial cell (AEC) mitochondria-regulated apoptosis in part by mechanisms involving p53-dependent transcription and a direct effect of p53 on the mitochondria (22). It is unknown whether p53 has a similar role in mediating PM-induced apoptosis.

Given the above findings, we reasoned that cross-talk between p53 and the mitochondria is important in causing AEC apoptosis after exposure to PM. We show that inhibitors of p53-dependent transcription (pifithrin- α , E6 overexpression, or dominant negative p53) prevent PM-induced apoptosis resulting from reductions in $\Delta\Psi_m$, caspase-9 activation, increased p53 mRNA and protein expression, and mitochondrial translocation of BAX and p53. A role for mitochondrial-derived ROS is suggested by our findings that mitochondrial dysfunction, apoptosis, and p53 protein expression are abolished in A549-p^o cells that are incapable of mitochondrial ROS production. Collectively, these data suggest a novel interaction between the mitochondria and p53 in mediating PM-induced AEC apoptosis, and that mitochondria-derived ROS have an important role in activating p53. Some of the results of these studies have been previously reported in the form of an abstract (23).

METHODS

PM and Reagents

The PM was collected by baghouse filter from ambient air in Düsseldorf, Germany, as previously described (10). The mass median aerodynamic diameter of the particles was $4.26 \pm 0.25 \mu\text{m}$. Unless stated otherwise, all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Cell Culture

A549 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with penicillin (100 U/ml), streptomycin (200 $\mu\text{g}/\text{ml}$), and 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY). For each experiment, we used a seeding density of 3.0×10^5 cells/ml/well plated onto six-well plates (Costar, Cambridge, MA). The cells were grown to 70% confluence over 24 h in a humidified 95% air–5% CO_2 incubator at 37°C, and then pretreated for 24 h in the presence of pifithrin- α (30 μM ; Sigma) in Dulbecco's modified Eagle's medium supplemented with 1% FBS. A549-p^o cells were prepared as previously described (24). Briefly, A549 cells were transfected with the human papillomavirus (HPV) type 16 E6 gene (designated as A549-E6). A549-E6 and empty vector control cells were a kind gift from Dr. K. J. Russell (University of Washington School of Medicine, Seattle, WA). The E6 oncoprotein of HPVs inactivates the cellular tumor suppressor p53 by binding and targeting it for degradation through the ubiquitin pathway (25). We also developed A549 cells that stably overexpress a dominant negative p53 gene (A549-GSE56) or empty vector control plasmid, as described previously (26). The GSE56 in pLXSN plasmid was a kind gift of Andrei Gudkov (Case Western Reserve University).

Rat alveolar type (AT) 2 cells were obtained, as previously described, using protocols that were approved by the Animal Care and Use Committee, Northwestern University (10, 27–29). The Northwestern University Institutional Review Board approved the use of human lung tissue obtained from the National Disease Research Interchange. Human AT2 cells were isolated from a lobe of human lung from four persons that had no evidence of injury (e.g., no consolidation or hemorrhage) and could be normally inflated. The pulmonary artery of this segment was perfused by lavaging the distal airspaces with Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline (PBS) solution containing ethyleneglycol-bis-(β -aminoethyl ether)- N,N' -tetraacetic acid and ethylenediaminetetraacetic acid. Cells were disaggregated by enzymatic digestion with the instillation of an elastase solution (8 U/ml; Worthington,

Lakewood, NJ) into the airspace of the chosen lung segment. Lung tissue was dissected from the airways, minced to 2-mm³ fragments in the presence of FBS, and filtered through layers of cotton gauze and 150- μm nylon mesh (Sefar America, Arden Hills, MN). After centrifugation, cells were layered onto a discontinuous Percoll density gradient and centrifuged to remove contaminating red blood cells. Cells accumulated at the interface represented a mix of AT2 cells and macrophages. Macrophage cells were depleted from the remaining cells by positive immunoselection, incubating the cells with anti-CD14-coated magnetic beads (DynaL Biotech, Carlsbad, CA) before magnetic selection, a technique that has no effect on the AT2 cells (29). The remaining AT2 cells were counted and cultured on collagen-coated plastic tissue culture dishes and glass coverslips. Cell viability was assessed by trypan blue exclusion. Cell purity was determined by immunofluorescence of cultured cells on subsequent days using cell-specific markers (27, 28). The percentage of type 2 cells ranged from 90 to 95%, with the major contaminating cells being macrophage cells and fibroblasts.

Mitochondrial Assays

AEC $\Delta\Psi_m$ and caspase-9 activation were assessed exactly as previously described by our laboratory (10, 28). Briefly, AEC $\Delta\Psi_m$ was based upon the percent difference in the ratio of tetramethylrhodamine ethyl ester and Mitotracker green (Molecular Probes, Eugene, OR) corrected for background fluorescence. Caspase-9 activity was assessed by a commercially available fluorometric assay kit following the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN). The data were normalized to the total protein concentration, as determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Apoptosis Assays

AEC apoptosis was assessed by annexin V staining (Roche Diagnostics) and DNA nucleosomal fragmentation ELISA, as previously described (27). Briefly, A549 cells were exposed to various doses of PM, and then the cells in the supernatant and attached to the dish were collected for determination of apoptosis. In some experiments, AECs were pretreated with pifithrin- α , as described *above*. Annexin V-stained cells were assessed under a fluorescence microscope (Eclipse TE200; Nikon, Melville, NY) by an investigator who was blinded to the experimental protocol.

ROS Assay

AEC ROS production was determined using 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes), as we previously described (24). After the cells were treated for 24 h with control media or PM (50 $\mu\text{g}/\text{cm}^2$), the cells were washed with PBS and then loaded for 30 min with 2',7'-dichlorodihydrofluorescein diacetate (10 μM) in Eagle's minimum essential medium without phenol red. After 4 h, the media were removed, the cells were lysed and centrifuged to eliminate debris, and the fluorescence in the supernatant was assessed using a fluorometer (excitation, 500 nm; emission, 530 nm). Data were normalized to A549 cells exposed to control media corrected per microgram of protein, as determined by the Bio-Rad protein assay.

Real-Time p53 mRNA Determination

Total RNA was extracted from PM-treated cells with or without pifithrin pretreatment using the RNeasy 4-polymerase chain reaction (PCR) kit from Ambion (Austin, TX) following the manufacturer's instructions exactly as previously described by our laboratory (22). The relative expression of p53 was normalized by the expression value of the 18s product using the comparative threshold method, as previously described (30).

Western Blot Analysis

Western blots were done by conventional techniques, previously described by our laboratory, using primary antibodies to p53 or Bax (Cell Signaling Technology, Beverly, MA) (22). For the cell fractionation experiments, cell mitochondrial and cytosolic proteins were isolated with ApoAlert Cell fractionation kit (Clontech, Mountain View, CA) following the manufacturer's recommendation. The same blots were stripped and probed with antibodies to β -tubulin (Santa Cruz Biotechnologies; Santa Cruz, CA) or cytochrome oxidase IV (Cell Signaling

Technology) to verify equal loading of cytosolic and mitochondrial protein, respectively. The protein bands were visualized by the enhanced chemiluminescence reaction system (GE Bioscience, Chiltern Hills, UK) according to the manufacturer's recommendation.

Immunohistochemistry

To determine whether PM induces p53 activation to distal lung cells, we used sections of paraffin-embedded lung tissue from CD57BL/6 mice instilled intratracheally with PBS alone (saline) or PM (1 or 100 μg) in PBS, as previously described by our laboratory, using a mouse monoclonal p53 primary antibody (Dako Corporation, Carpinteria, CA) (22, 31). The Animal Care and Use Committee, Northwestern University, approved our mouse model of PM-induced lung damage.

TUNEL-stained Mouse Lungs

Lung tissue sections from CD57BL/6 mice instilled intratracheally with PBS alone (saline) or PM (1, 10, 100, or 200 μg) in PBS were deparaffinized, permeabilized, labeled with fluorescein-FragEL terminal deoxynucleotidyl transferase (TdT) labeling mix, and treated with TdT using the ApopTag Plus Fluorescein *in situ* Apoptosis Detection kit (Chemicon, Temecula, CA) according to the manufacturer's directions, as previously described (27). A positive control was generated by treating a lung section with DNase (1 $\mu\text{g}/\mu\text{l}$; Sigma-Aldrich, St. Louis, MO) for 20 min at room temperature before staining. A negative control was generated by treating a lung section with reaction buffer lacking TdT. The sections were then washed twice with PBS and fixed in Bouin's solution for 7 min at room temperature, washed three times in PBS, and treated with 0.1% Triton X-100 for 30 min at 37°C. The cells were then washed three times with PBS and blocked with normal goat serum (Sigma-Aldrich) for 30 min at 37°C. The cells were then incubated with an antibody to the 8.1.1 protein (University of Iowa Hybridoma Bank) diluted in normal goat serum (1:100). This antibody recognizes the RT1 epitope on the membrane of AT1 cells. The sections were then washed twice with PBS and treated with a rhodamine-labeled goat anti-hamster secondary antibody (1:200; Molecular Probes) for 30 min at 37°C, and washed twice with 0.05% Tween and twice with PBS before mounting with gelvatol. Terminal deoxynucleotidyl transferase-mediated deoxyuridine-5'-triphosphate-biotin nick end labeling (TUNEL)-positive nuclei were counted in approximately 15 high-power fields (400 $\times$) per section using an Eclipse TE200 microscope (Nikon). Representative images were photographed from the same sections using an LSM 510 Laser Scanning Confocal microscope (Carl Zeiss, Minneapolis, MN).

Statistical Analysis

All data are expressed as the means ($\pm$ SEM). An unpaired Student's *t* test was used to assess the difference between two groups. One-way analysis of variance was performed when more than two groups were compared with a single control, and then differences between individual groups within the set were assessed by Tukey's multiple comparison test when the *F* statistic was less than 0.05. A *p* value of less than 0.05 was considered significant.

RESULTS

Inhibitors of p53-dependent Transcription Prevent PM-induced Mitochondrial Dysfunction and Apoptosis

To determine whether p53-dependent transcription is required for PM-induced AEC mitochondrial dysfunction and apoptosis, we assessed the protective effects of a pharmacologic p53 inhibitor (pifithrin- α). Pifithrin- α is a small, water-soluble compound that blocks p53 DNA binding in cells exposed to doxorubicin, ultraviolet light, or γ radiation (32). In these studies, we used both a human adenocarcinoma cell line (A549), which has AT2-like features and normal p53 function, as well as primary AT2 cells isolated from rats and from human lungs deemed unsuitable

Figure 1. Particulate matter (PM)-induced mitochondrial membrane potential change and caspase-9 activation are inhibited by pifithrin- α . A549 cells were treated with pifithrin- α (30 μM $\times$ 24 h), washed, exposed 4 h to PM (0–50 $\mu\text{g}/\text{cm}^2$), and then (A) mitochondrial membrane potential change ($\Delta\Psi\text{m}$) and (B) caspase-9 activation were determined as described in METHODS. PM caused dose-dependent $\Delta\Psi\text{m}$ and caspase-9 activation in A549 cells, and these effects were blocked by pifithrin- α . Control caspase-9 activation was 16.6 ± 0.4 U/mg protein. Data are expressed as the mean $\pm$ SEM; **p* < 0.05 vs. control; †*p* < 0.05 vs. no pifithrin- α ; *n* = 4.

for transplantation. As shown in Figure 1, exposure of A549 cells to PM (0–50 $\mu\text{g}/\text{cm}^2$) for 24 h exhibited a dose-dependent reduction in $\Delta\Psi\text{m}$ and caspase-9 activation that were consistent with our prior report (10). Notably, pifithrin- α (30 μM) completely blocked the deleterious effects of PM on mitochondrial function (Figure 1). Pifithrin- α also abolished PM-induced apoptosis, as assessed by both annexin V binding and DNA fragmentation (Figure 2). In primary human and rat AT2 cells, treatment with pifithrin- α also prevented PM-induced caspase-9 activation and apoptosis (Figure 3).

We also used A549-E6 cells to investigate a genetic approach to inhibit p53 transcriptional activity that depletes cellular p53 by enhancing its degradation via ubiquitination (25). As compared to A549 empty vector control cells, A549-E6 cells were protected against PM-induced caspase-9 activation and DNA fragmentation (Figure 4). Similarly, A549-GSE56 blocked PM-induced caspase-9 activation as well as DNA fragmentation (Figure 4). Collectively, these findings in A549-E6 cells and A549 cells that overexpress a dominant negative p53 gene are in accordance with our pifithrin- α data described *above* and firmly implicate a requirement for p53-dependent transcription in PM-induced AEC apoptosis.

PM Augments A549 Cell p53 mRNA and Protein Expression, and These Effects Are Inhibited by Pifithrin- α

To assess whether PM induces p53 mRNA expression, we used real-time reverse transcriptase-PCR (RT-PCR). A549 cells were treated with control media or pifithrin- α , as noted previously here, and then the relative PM-induced p53 mRNA abundance,

Figure 2. PM-induced apoptosis is suppressed by pifithrin- α . A549 cells were treated with pifithrin- α , as in Figure 1, exposed 24 h to PM (0–50 $\mu\text{g}/\text{cm}^2$), and apoptosis was assessed by both (A) annexin V binding and (B) DNA fragmentation as described in METHODS. Pifithrin- α blocks both PM-dependent annexin V binding as well as DNA fragmentation. Control DNA fragmentation was 117.0 ± 1.4 U/mg protein. Data are expressed as the mean $\pm$ SEM; * $p < 0.05$ vs. control; † $p < 0.05$ vs. (–) pifithrin; $n = 4$.

corrected for 18s mRNA, was determined at various time points. As shown in Figure 5, treatment with PM (50 $\mu\text{g}/\text{cm}^2$) significantly increased p53 mRNA levels at 24 h by approximately eightfold ($p < 0.05$ vs. control). Although there was a trend toward PM increasing p53 mRNA levels at 8 h (~twofold), it did not reach statistical significance. Furthermore, negligible changes in PM-induced p53 mRNA levels were detected at earlier time periods (Figure 5). Consistent with the protective effects of pifithrin- α against PM-induced AEC mitochondrial dysfunction and apoptosis, pifithrin- α completely blocked PM-induced p53 mRNA accumulation at 24 h (Figure 5C). In accordance with these results, p53 protein abundance, as measured by immunoblotting of cell lysates, was increased at 24 h after PM exposure, and pifithrin- α abolished this effect (Figure 6).

PM Induces Bax and p53 Mitochondrial Translocation, and This Effect Is Prevented by p53 Inactivation by Pifithrin- α Pretreatment

One mechanism by which p53 triggers mitochondrial-dependent apoptosis in response to DNA damaging agents is by augmenting Bax and p53 mitochondrial translocation and subsequent mitochondrial permeabilization (17–19, 22, 33). To examine whether this occurs after PM exposure, A549 cells were treated as described previously here, and then subcellular protein fractions from the cytosol and the mitochondria were assessed by Western analysis, and the levels of Bax and p53 were expressed relative to cytochrome oxidase IV (mitochondrial protein) or β -tubulin (cytosolic protein). Compared with controls, PM increased mitochondria-targeted Bax and p53 protein levels by approximately three- and twofold, respectively (Figure 7). Notably, pifithrin- α blocked PM-induced mitochondrial translocation of p53 and Bax. These data demonstrate that p53-dependent transcriptional activity is required for the translocation of Bax from the cytosol to the mitochondria, as well as for the direct mitochondrial effects of p53.

A549 ρ^0 Cells That Are Incapable of Mitochondrial ROS Production Are Resistant to PM-induced $\Delta\psi_m$, p53 Expression, and Apoptosis

To examine the role of mitochondrial-derived ROS in mediating PM-induced AEC mitochondrial dysfunction and apoptosis, we used A549 cells that lack mitochondrial DNA and a functional mitochondrial electron transport chain for oxidative phosphorylation (A549- ρ^0) that we have previously shown are incapable of mitochondrial ROS production or asbestos-induced p53 promoter activity (22, 24). We exposed wild-type A549 and A549- ρ^0 cells to PM (50 $\mu\text{g}/\text{cm}^2$) for 24 h, and then measured PM-induced ROS production, $\Delta\psi_m$, p53 expression, and apoptosis (DNA fragmentation). As shown in Figure 8, A549- ρ^0 cells completely blocked PM-induced ROS production, $\Delta\psi_m$, p53 protein expression, and DNA fragmentation. These findings suggest that PM-induced AEC apoptosis regulated by p53 is triggered, at least in part, by ROS originating from the mitochondria.

PM Augments p53 Expression and Apoptosis in Cells at the Bronchoalveolar Duct and Alveolar Regions

Previous studies have established that rodent lungs exposed to particulates, such as asbestos and silica, have increased expression of p53 and apoptosis (22, 27, 34, 35). To determine whether PM augments lung p53 protein accumulation, we performed immunohistochemistry on mouse lungs 72 h after a single intratracheal instillation of saline (50 μl) or PM (1–200 μg in 50 μl).

Figure 3. Pifithrin- α blocks PM-induced DNA fragmentation in primary isolated AT2 cells from humans and rats. Primary isolated human ($n = 4$) and rat AT2 ($n = 6$) cells were obtained as described in METHODS. The cells were then treated with pifithrin- α , as described in Figure 1, and PM-induced DNA fragmentation after 24 h was assessed as described in METHODS. PM increased DNA fragmentation in both rat (solid bars) and human (gray bars) AT2 cells, and these effects were blocked by pifithrin- α . Control DNA fragmentation in human and rat AT2 cells was 98.4 ± 3.2 U/mg protein and 112.0 ± 2.0 U/mg protein, respectively. * $p < 0.05$ vs. control; † $p < 0.05$ vs. PM alone.

Figure 4. A549-E6 cells do not undergo PM-induced caspase-9 activation or apoptosis. A549-E6 and control A549 empty vector transfected cells were exposed to PM for 24 h, and then (A) caspase-9 activation and apoptosis as assessed by (B) DNA fragmentation were determined as described in METHODS. Unlike control cells, PM-induced caspase-9 activation and DNA fragmentation were completely abolished in the A549-E6 cells. A549-GSE56 that express a dominant negative p53 gene and control A549 empty vector transfected cells were treated with PM for 24 h, and then (C) caspase-9 activation and apoptosis, as assessed by (D) DNA fragmentation, were determined as described in METHODS. Unlike control cells, PM-induced caspase-9 activation and DNA fragmentation were completely abolished in the A549-p53 dominant/negative cells. Control A549 cell caspase-9 activation was 138.0 ± 5.7 U/mg protein, whereas DNA fragmentation was 144.0 ± 11.2 U/mg protein. Data are expressed as mean $\pm$ SEM; *p < 0.05 control vs. PM; †p < 0.05 E6 vs. empty vector with same dose of PM; n = 4.

Figure 5. Pifithrin- α blocks increases in PM-induced p53 mRNA levels. A549 cells were exposed to PM (50 $\mu\text{g}/\text{cm}^2$), and p53 mRNA was determined by reverse transcriptase-polymerase chain reaction (RT-PCR), as described in METHODS. PM significantly induced p53 mRNA expression by 24 h, as shown in a representative RT-PCR blot (A) and quantitated by densitometry (B). PM (50 $\mu\text{g}/\text{cm}^2$)-induced p53 mRNA accumulation at 24 h was abolished by pifithrin- α (C). *p < 0.05 vs. control; †p < 0.05 vs. (-) pifithrin; n = 3.

As seen in Figure 9, under control conditions or low-dose PM instillation, only rare p53 immunostained cells were noted at the bronchoalveolar duct junctions (arrows). In contrast, more generalized increases in p53 expression were noted after exposure to 100 μg of PM (Figure 9). PM also induced dose-dependent apoptosis in cells at the bronchoalveolar duct and alveolar regions, as assessed by TUNEL-stained nuclear morphology (Figure 10). Although comprehensive cell-specific identification studies were not performed, p53 and TUNEL staining were evident in AECs (both AT1 cells, as identified by the AT1 cell marker, 8.1.1, and, perhaps, AT2 cells) and macrophages (identified morphologically in the alveolar space), similar to what has been reported for other inhaled agents, such as silica and asbestos (22, 34, 35).

DISCUSSION

Activation of p53 is an important mechanism that prevents malignant transformation by genotoxic stress. Although prospective epidemiologic human studies have established that PM exposure increases the adjusted risk ratio for developing lung cancer by approximately 1.10 per increment of 10 $\mu\text{g}/\text{m}^3$ (4–7), the mechanisms involved have not been established. In this study, we show that inhibitors of p53-dependent transcription (pifithrin- α , E6 overexpression, or dominant negative p53 gene expression) block PM-induced increases in p53 mRNA and protein expression, mitochondrial translocation of Bax and p53, as well as subsequent mitochondria-regulated AEC apoptosis. Furthermore, A549-p53 cells that are incapable of mitochondrial ROS production are protected against PM-induced ROS production, $\Delta\Psi\text{m}$, p53 protein expression, and apoptosis. Finally, we provide *in vivo* evidence that PM increases p53 protein expression and apoptosis in cells at the bronchoalveolar duct junctions in mouse lungs. Collectively, these data strongly implicate p53-dependent transcriptional mechanisms as a cause of PM-induced AEC mitochondrial dysfunction and apoptosis that are due, in part, to mitochondria-derived ROS production and the mitochondrial translocation of Bax and p53.

Although p53 stimulates apoptosis by complex mechanisms, we focused on the mitochondria, because others, as well as our group, have established that airborne exposures that cause lung

Figure 6. PM-induced p53 protein expression is blocked by pifithrin- α . A549 cells were treated with pifithrin, as described in Figure 1, then exposed to PM (50 $\mu\text{g}/\text{cm}^2$) for various time periods, and p53 protein abundance was determined by Western analysis as described in METHODS. The relative abundance of p53 protein under each condition was expressed relative to β -tubulin. PM induced p53 protein expression at 24 h, and this was blocked by pifithrin- α . * $p < 0.05$ vs. control; † $p < 0.05$ vs. (–) pifithrin; $n = 3$.

cancer—for example, PM and asbestos, but not inert particulates (e.g., glass beads or titanium dioxide)—induce apoptosis by activating the mitochondria-dependent death pathway (9, 10, 28). Moreover, we detect negligible levels of caspase-8 (the death receptor activated caspase) in our system after exposure to either PM or asbestos (10, 28). One of the key findings of this study is that inhibitors of p53-dependent transcriptional activity (pifithrin- α , E6 protein overexpression, or p53 dominant negative gene expression) blocked PM-induced mitochondrial dysfunction, as assessed by $\Delta\psi_m$ and caspase-9 activation, as well as apoptosis, as assessed by annexin V staining and DNA fragmentation (Figures 1–4). Furthermore, the protective effects noted in A549-E6 cells exposed to PM in the current study are comparable to the beneficial effects observed against radiation- and asbestos-induced cytotoxicity (22, 25).

In this study, we show that PM caused comparable effects in A549 cells, a malignant line of bronchoalveolar cells with AT2-

like features and a wild-type p53 function, and primary isolated AT2 cells from rats (Figure 3). In addition, this is the first report, to our knowledge, that primary AT2 cells isolated from human lungs undergo apoptosis in response to PM, and that this apoptosis requires p53 transcriptional activity (Figure 3). These findings are in accordance with our earlier work showing that PM and asbestos cause comparable levels of DNA strand breaks, mitochondrial dysfunction, and apoptosis in AECs (10, 27, 28). We report here that PM increased p53 mRNA levels nearly eightfold at 24 h, as assessed by real-time RT-PCR (Figure 5), whereas p53 protein levels increased by nearly 60%, as assessed by Western analysis (Figure 6). Notably, pifithrin- α (Figures 5 and 6) blocked PM-induced p53 mRNA and protein expression as well as subsequent PM-induced AEC mitochondrial dysfunction and apoptosis (Figures 1–4). To our knowledge, this is the first evidence linking PM exposure to p53-dependent apoptosis in lung epithelial cells.

Figure 7. PM-induced A549 cell mitochondrial Bax and p53 translocation is suppressed by pifithrin. A549 cells were exposed to PM (50 $\mu\text{g}/\text{cm}^2$) in the presence or absence of pifithrin- α (pif) for 24 h, and then mitochondrial and cytosolic protein were obtained and assessed for Bax and p53 protein by Western analysis. The differences observed in the levels of Bax and p53 from three experiments are shown in a densitometric analysis of mitochondrial (m) and cytosolic (c) proteins. The levels of cytochrome oxidase IV (COX IV) were used to confirm the presence of mitochondrial protein as well as comparable loading. β -Tubulin was used to ensure equal loading of cytosolic protein (data not shown). * $p < 0.05$ vs. control; † $p < 0.05$ vs. (–) pifithrin; $n = 3$.

Figure 8. PM-induced mitochondrial dysfunction, apoptosis, and p53 accumulation are abolished in A549-p° cells. A549-p° and wild-type A549 cells were exposed 24 h to PM (50 μg/cm²), and reactive oxygen species (ROS) production assessed by dichlorodihydrofluorescein (DCF) fluorescence (A), $\Delta\Psi_m$ (B), DNA fragmentation (C), and p53 expression (D) were determined. Unlike control cells, A549-p° cells were resistant to PM-induced ROS production, $\Delta\Psi_m$, p53 expression, and DNA fragmentation. Control A549 cell DNA fragmentation was 24.8 ± 0.7 U/mg protein. * $p < 0.05$ vs. control; † $p < 0.05$ A549-p° vs. A549 cells with same dose of PM; $n = 4$.

It is established that ROS can activate p53 expression, and that p53-induced apoptosis involves the generation of ROS, but it is unknown whether PM-induced p53 expression and mitochondria-regulated apoptosis are similarly affected (20, 21, 36). Two lines of evidence, including data presented in the current study, firmly implicate ROS from the mitochondria in mediating PM-induced p53 expression and subsequent mitochondria-regulated apoptosis. First, previous studies by others, as well as

our group, showing protection by antioxidants (N-acetylcysteine or sodium benzoate), iron chelators (phytic acid or deferoxamine), or in cells that overexpress a mitochondrial apoptosis regulator, Bcl-2 or Bcl-xl, suggest that one mechanism by which PM causes apoptosis is the generation of ROS from the mitochondria (9, 10). There is evidence that organic chemicals in diesel exhaust particles contribute to free radical formation and mitochondrial dysfunction (9). Second, in this study, we showed

Figure 9. PM induces p53 immunohistochemical staining of cells at the bronchoalveolar duct junctions and alveoli. Paraffin-embedded mouse lungs were analyzed for p53 protein expression by immunohistochemistry 72 h after a single intratracheal instillation of saline or PM (1 or 100 μg in 50 μl), as described in METHODS, at 100× and 1,000× magnification. Under control conditions or low-dose PM instillation, only rare p53 immunostained cells were noted at the bronchoalveolar duct junctions (arrows). In contrast, high-dose PM induced marked expression of p53 throughout the distal lungs.

Figure 10. PM induces TUNEL staining of cells at the bronchoalveolar duct junctions and alveoli. Paraffin-embedded mouse lungs were analyzed for TUNEL staining 24 h after a single intratracheal instillation of saline or PM (1, 10, 100, or 200 µg in 50 µl), as described in METHODS. The sections were counterstained with the 8.1.1 antibody that recognizes an epitope on the membrane of ATI cells. Under control conditions or low-dose PM instillation, only rare TUNEL-stained cells were noted at the bronchoalveolar duct junctions. In contrast, high-dose PM induced higher levels of TUNEL staining throughout the distal lungs. * $p < 0.05$ vs. control; $n = 4$.

that A549- ρ^0 cells that are incapable of mitochondrial ROS production are protected against PM-induced AEC ROS production, p53 expression, mitochondrial dysfunction, and apoptosis (Figure 8). These findings concur with both the observation that ρ^0 human fibroblasts do not increase p53 expression caused by oxidative stress (37, 38) and that of our previous study, showing that asbestos induces significantly less oxidative stress, mitochondrial dysfunction, p53 promoter activity, and apoptosis in A549- ρ^0 cells (22, 24). Although hydroxyl radical formation in the mitochondria was not directly demonstrated in the present study, this seems likely based upon the protective effects in A549- ρ^0 cells plus the very highly reactive nature of hydroxyl radicals that prevents them from traveling beyond the site of their formation (39). Collectively, these data suggest a novel mechanism involving mitochondria-derived ROS in activating p53 expression after PM exposure, which results in mitochondria-regulated AEC apoptosis.

The mechanisms by which p53 regulates mitochondria-regulated apoptosis are complex and incompletely understood, but some established pathways include effects on the Bcl-2 family of anti- and proapoptotic proteins via p53-dependent transcription mechanisms as well as direct effects of p53 on the mitochondria (1, 2). In this study, we found that PM exposure causes Bax and p53 mitochondrial translocation (Figure 7). Moreover, the observation that pifithrin- α prevents PM-induced AEC mitochondrial translocation of Bax and p53 suggests that p53-dependent transcription is important in mediating the direct effects of p53 on the mitochondria-dependent apoptosis from

PM exposure (Figures 1 and 7). These data on PM are consistent with studies demonstrating that DNA-damaging agents activate p53-dependent mechanisms that induce Bax translocation to the mitochondria (17–22). It is conceivable that other proapoptotic pathways may be involved in our model, as Bax is only one of several p53-dependent transcriptional targets that have been implicated in promoting apoptosis by the intrinsic pathway (e.g., Bax, Bak, p53-upregulated modulator of apoptosis, and others) (1, 2). In addition to the BH3-only-like activity of p53, p53 also inhibits the transcription of antiapoptotic Bcl2 family members, such as Bcl-2 and Bcl-xl (1, 2). We previously reported that A549 cells overexpressing Bcl-xl are protected against PM-induced mitochondria-regulated apoptosis (10). A hypothetical model, depicting the oxidant-dependent mechanisms by which PM affects p53 expression and mitochondria-regulated apoptosis, is shown in Figure 11. Additional studies are warranted to further elucidate the molecular mechanisms regulating PM-induced AEC p53 expression that dictates whether cells undergo DNA repair/survival, apoptosis, or malignant transformation.

The *in vivo* relevance of our findings was investigated by assessing p53 expression and TUNEL staining in cells at the bronchoalveolar duct junctions of mice exposed to a single intratracheal instillation of PM using a murine model that we have previously described (31). In this model, a single intratracheal instillation of PM causes dose-dependent reductions in alveolar edema clearance that is associated with reductions in Na/K-ATPase and minimal histologic evidence of pulmonary injury after 24 h. In the current study, we have shown that PM, unlike

Figure 11. Hypothetical model of alveolar epithelial cell (AEC) PM-induced apoptosis. After PM internalization into AECs, p53 activation is induced through mitochondria ROS-dependent and independent mechanisms. P53 can also increase mitochondrial ROS production. PM-induced AEC apoptosis by the mitochondria-regulated death pathway is triggered by p53-dependent transcription of proapoptotic family members, as well as a direct effect of p53 on the mitochondria.

saline controls, increased p53 expression, as assessed by immunohistochemistry (Figure 9), and apoptosis, as assessed by TUNEL staining (Figure 10). p53 and TUNEL staining were evident in cells along the bronchoalveolar duct junctions, including AECs (both AT1 and, perhaps, AT2 cells). The dose of PM that we used in our *in vivo* model is within the range of human particulate exposure that can be inhaled ($\leq 10 \mu\text{m}$). In 2002, the U.S. Environmental Protection Agency reported a range of maximal city concentrations of particulate matter smaller than $10 \mu\text{m}$ in diameter ($26\text{--}534 \mu\text{g}/\text{m}^3$) (40). Our findings concur with studies in humans exposed to various types of air pollutants showing increased p53 gene expression in lung cancers, mutations in the p53 gene in nonmalignant epithelial cells from the sputum of individuals without evidence of lung cancer, and p53 protein expression in the nasal epithelium of dysplastic lesions (41–44). Our data implicating p53 in mediating PM-induced AEC apoptosis are consistent with a recent study showing that silica, which is another particle capable of causing lung cancer, failed to induce apoptosis in lung cells of p53 knockout animals, but did so in p53 wild-type mice (35).

An important consideration in this study is the use of a pharmacologic inhibitor, pifithrin- α . Although pifithrin- α was initially isolated as a specific inhibitor of p53-dependent transcription (32), more recent evidence shows that it can also suppress heat shock and glucocorticoid signaling pathways (45). We also employed a genetic approach to deplete p53 by using A549-E6 cells, as others have shown contain a functionally inactive p53 gene product by targeting p53 for ubiquitination and are resistant to radiation-induced G1 checkpoint control necessary for radiation-induced cell cycle arrest (22, 25). Although E6 can target additional proteins for ubiquitin-mediated degradation via the ubi-

quitin ligase protein, E6-AP (e.g., E6TP1, hScrib, hDIg, and Bak), a critical role for E6-AP-induced p53 degradation in causing the effects of E6 in HPV-positive cells was demonstrated using RNA interference for p53 (46, 47). The concordance of our findings with pifithrin- α , A549-E6 cells, or A549 cells that overexpress a p53 dominant negative gene in multiple assays assessing PM-induced mitochondrial function, p53 mRNA and protein expression, and apoptosis firmly supports the important regulatory role of p53 in our model.

In conclusion, we have shown an important role for p53-dependent transcription mechanisms in mediating PM-induced AEC mitochondria-regulated apoptosis. Our prior report demonstrating protection by iron chelators and free radical scavengers, combined with the current study, strongly implicates ROS from the mitochondria, as well as Bax and p53 mitochondrial translocation, in promoting mitochondria-regulated apoptosis (10). We also found that PM causes p53 expression and apoptosis (TUNEL staining) in cells at the bronchoalveolar duct junctions and alveoli of mice. A hypothetical model by which PM mediates AEC mitochondrial dysfunction and apoptosis is summarized in Figure 11. We reason that the interactive effects between p53 and the mitochondria have a key role in determining AEC survival and/or malignant transformation after PM exposure that is important in mediating the pathophysiologic effects of PM that promote pulmonary toxicity (1, 2, 48, 49). Excessive apoptosis may facilitate airway remodeling, whereas inadequate apoptotic mechanisms may promote the formation of a malignant clone of cells harboring mutated DNA. Furthermore, the mitochondrial DNA may be an especially important target given the recent observation that p53 augments the incorporation step of base excision DNA repair in the mitochondria (50), and that mitochondrial DNA, as compared with nuclear DNA, is more susceptible to oxidative DNA damage and acquires mutations at a 10-fold higher rate (51). Thus, strategies aimed at reducing PM-induced mitochondrial ROS production, as well as mitochondrial DNA damage, should maintain pulmonary epithelial cell barrier function and, thereby, prevent airway remodeling and malignant transformation.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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