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Air pollution induces enhanced mitochondrial oxidative stress in cystic fibrosis airway epithelium

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Abstract **We studied the effects of airborne particulate matters (PM) on cystic fibrosis (CF) epithelium. We noted that PM enhanced human CF bronchial epithelial apoptosis, activated caspase-9 and PARP-1; and reduced mitochondrial membrane potential. Mitochondrial inhibitors (4,4-diisothiocyanatostilbene-2,2′disulfonic acid, rotenone and thenoyltrifluoroacetone) blocked PM-induced generation of reactive oxygen species and apoptosis. PM upregulated pro-apoptotic Bad, Bax, p53 and p21; and enhanced mitochondrial localization of Bax. The anti-apoptotic Bcl-2, Bcl-xl, Mcl-1 and Xiap remained unchanged; however, overexpression of Bcl-xl blocked PM-induced apoptosis. Accordingly, we provide the evidence that PM enhances oxidative stress and mitochondrial signaling mediated apoptosis via the modulation of Bcl family proteins in CF.**
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Keywords: Ambient air pollution particle; Apoptosis; Cystic fibrosis; Mitochondria; Particulate matter; Oxidative stress

1. Introduction

Airborne particulate matter (PM 2.5 μm) increases morbidity and mortality from cardiopulmonary diseases resulting in an estimated 500 000 deaths each year worldwide [1]. Accumulating evidence show that there is a consistent positive association between the elevated levels of air pollution and worsening of symptomatic pulmonary disease, specifically in a potentially sensitive group with preexisting pulmonary diseases such as asthma, chronic obstructive pulmonary disease and cystic fibrosis (CF) [2–5]. CF is the most common lethal genetic diseases affecting Caucasians, with an incidence of approximately 1 in 2000 to 1 in 3200 live births [3–5]. The clinical course of this disease includes continued inflammation, acute on chronic pulmonary infections and malnutrition. However, an accelerated decline in lung function reflects poor prognosis, resulting in up to 92% of deaths due to cardiopulmonary failure [3–5]. Although, several environmental factors are known to affect the prognosis in preexisting pulmonary diseases; in patients with CF, annual average exposure to particulate air pollution was shown to be associated with an increased risk of pulmonary exacerbations and a decline in lung function [4].

PM is genotoxic to lung epithelium; however, identification of the key mediators that enhance the effect of PM in preexisting pulmonary diseases, such as CF, yet remains elusive [6]. PM is a potent stimulus of mitochondrial dysfunction [6–9]. PM is known to modulate the Bcl-2 family proteins which consist of both pro- and anti-apoptotic members that act as gatekeepers and regulate the translocation of death-promoting molecules from the mitochondria [8,10,11]. Particularly, Bax, a pro-apoptotic Bcl-2, translocates to the mitochondrial and initiates mitochondrial dysfunction leading to apoptosis [12,13]. Disruption of mitochondrial electron transport can further augment reactive oxygen species (ROS) production and amplify an apoptotic stimulus due to release of mitochondrial and non-mitochondrial derived ROS [12–16]. However, the molecular mechanisms underlying the effects of PM in preexisting pulmonary diseases, such as, CF, are not clearly defined. In this study, we determine whether enhanced mitochondrial signaling and oxidative stress mediate PM-induced apoptosis in human CF bronchial epithelium.

2. Materials and methods

2.1. Materials

The PM used in our study is well characterized ambient particle 2.5 μm with known elemental analyses as described in our previous study [6,17]. The particles were aerosolized from a turntable into a small-scale powder disperser (TSI Inc., St. Paul, MN) utilizing a high airflow to break up aggregates in the venturi throat. The outlet of the aerosol generator was attached directly to an aerodynamic particle sizer (TSI Inc.) and the aerosol was sampled on four occasions for 20 s. Data were expressed as the average mass median aerodynamic diameter from the four replicate samples. Elemental analyses of the PM were accomplished by infrared or thermal conductivity assays (Galbraith Labs, TN). All other chemicals were purchased from Sigma Chemicals.

2.2. Cell culture

IB3-1 and S-9 CF human bronchial epithelial cells were purchased from American Type Culture Collection. IB3-1 cells contain compound heterozygote delta F508 and W1282X non-sense CF transmembrane conductance regulator (CFTR) mutations. The S9 cells are derived from the IB3-1 cell line with the CF phenotype corrected by transfection with wild-type adeno-associated viral CFTR. The S9 cells stably express wild-type CF transmembrane conductance regulator and they are used as control cells. The cells were cultured in precoated dishes with a mixture of 0.01 mg/ml fibronectin, 0.03 mg/ml bovine

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Abbreviations: AEC, alveolar epithelial cells; BAD, Bcl2-antagonist of cell death; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4′-diisothiocyanatostilbene-2,2′disulfonic acid; NHBE, normal human bronchial epithelial cells; PM, particulate Matter; ROS, reactive oxygen species; TTFA, thenoyltrifluoroacetone

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collagen type I and 0.01 mg/ml bovine serum albumin dissolved in LHC-8 Basal Medium (ATCC) with 5% fetal bovine serum in a humidified 95% air–5% CO_2 incubator at 37 °C. The human bronchial epithelial cells (HBEC) were purchased from ATCC. The cells were grown using above condition in ATCC complete growth medium using Base medium BEBM (Clonetics Corporation) with 0.5 ng/ml recombinant epidermal growth factor, 500 ng/ml hydrocortisone, 0.005 mg/ml insulin, 0.035 mg/ml bovine pituitary extract, 500 nM ethanolamine, 500 nM phosphoethanolamine, 0.01 mg/ml transferring, 6.5 ng/ml 3,3′,5-triiodothyronine, 500 ng/ml epinephrine and 0.1 ng/ml retinoic acid. Overexpression of Bcl-xl: the cells were transfected with the full-length human Bcl-xl gene cloned into plasmid pCDNA-3 and subcloned into pIRES1-neo vector (Clontech) as previously described in other cells. The cells were transfected using cationic lipid LipofectAMINE Plus (Life Technologies) according to the manufacture's specifications and transfected clones were identified based upon growth in Geneticin (1 mg/ml; Life Technologies)-containing media. Controls consisted of cells transfected with the geneticin-resistance gene but without Bcl-xl. Enhanced expression of Bcl-xl protein levels in the bcl-xl-treated cells, but not the empty vector controls, were confirmed by Western analysis (data not shown).

2.3. Apoptosis assay

S9 and IB-3 cell apoptosis was assessed by DNA nucleosomal fragmentation ELISA (Roche Diagnostics, Indianapolis, IN) as previously described [6,17].

2.4. MTT cell viability assay

Cells were treated with PM and then incubated with MTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt) (20 μl) for 3 h. An absorbance at 490 nm was measured to quantify the formazan product.

2.5. Mitochondrial membrane potential change (ΨΔm)

The ΨΔm was assessed by a fluorometric assay using tetremethylrhodamine ethyl ester (TMRE) or Mitotracker green (Molecular Probes) followed by carbonyl cyanide trifluoromethoxyphenlhydrazone (FCCP) as previously described [6].

2.6. Caspase activity assays

Caspases 9 and 3 activities were assessed by using colorimetric activity assay kits from Upstate laboratory as per the manufacturer's protocol [6].

2.7. Western blot analysis

Proteins were size fractionated by 10% gel electrophoresis and transferred to nitrocellulose membranes using a semi-dry transfer (Bio-Rad). Blots were incubated with specific antibodies overnight at 4 °C and developed with an enhanced chemiluminescence detection kit (Amersham).

2.8. Immunofluorescence staining

Cells were treated with PM and then incubated with 1 μM Mitotracker Red to stain the mitochondria. The slides were rinsed, fixed and then incubated overnight with a specific antibody (Bax: cell signaling technology) followed by DAPI for nuclear staining.

2.9. Dichlorofluorescein assay

The production of ROS was determined using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes). Cells were treated with PM and then loaded with DCFH-DA (10 μM) for 30 min. The fluorescence was measured at excitation 500 nm/emission 530 nm.

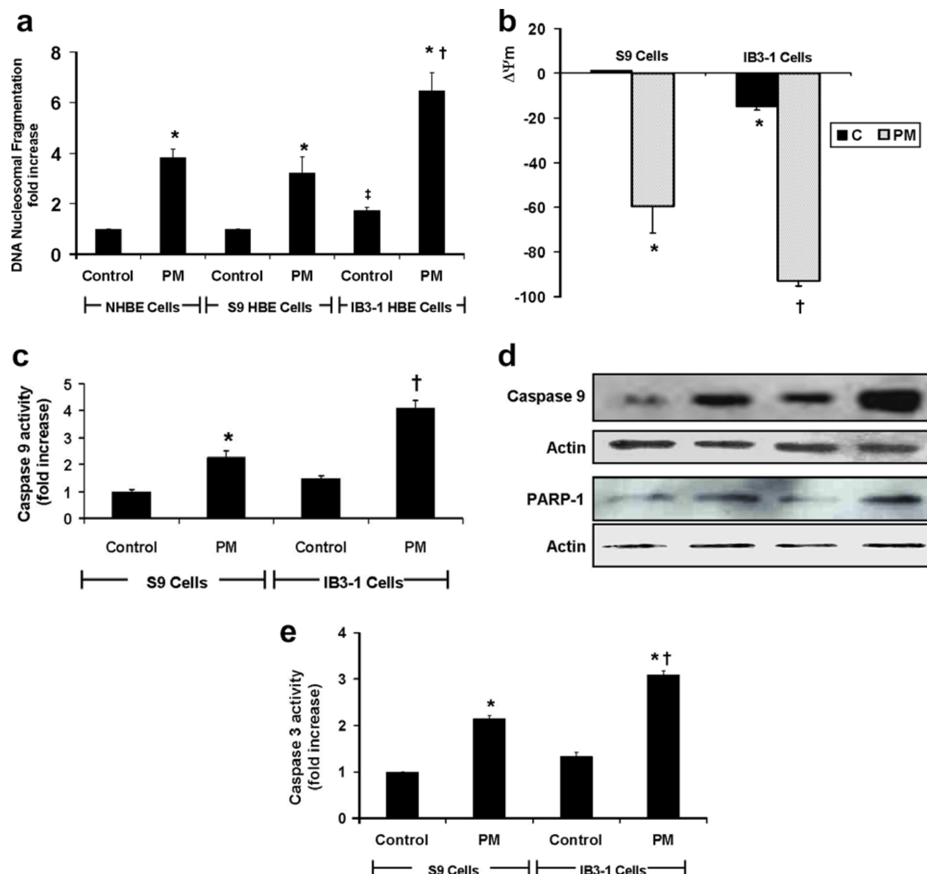

Fig. 1. PM enhanced apoptosis in human CF bronchial epithelium via mitochondrial pathway: PM (25 μg/cm^2) caused sixfold increase in DNA fragmentation in IB3-1 cells as compare to 3.5-fold in the S9 and normal HBE cells (Fig. 1a). PM induced significant reduction in mitochondrial membrane potential and caused caspase-9, caspase-3 and PARP-1 activation in IB3-1 cells as compare to S9 cells, suggesting enhanced mitochondrial signaling in CF (Fig. 1b–e). Mean ± S.E.M., $^*P < 0.05$ control vs. PM, $^{\dagger}P < 0.05$ S9 cells vs. IB3-1 cells PM, $^{\ddagger}P < 0.05$ S9 cells control vs. IB3-1 cells control and $n = 3$.

2.10. Statistics

Data is reported as mean ± S.E.M. Statistical analysis was done by one-way ANOVA and Tukey tests. Results were considered significant when $P < 0.05$.

3. Results and discussion

Mitochondria are known to be the critical mediators of PM-induced oxidative stress. Mitochondrial signaling is regulated by the anti- and pro-apoptotic members of the Bcl-2 family which are mandatory for the initiation of apoptosis [12–17]. Accumulation and translocation of pro-apoptotic proteins to the mitochondria enhances apoptosis through oxidative stress [18–20]. Given the important role of mitochondrial signaling in PM-induced cytotoxicity, we determined whether enhanced oxidative stress and mitochondrial signaling mediate the effects of PM in CF epithelium.

In this study, we demonstrate that PM induces oxidative stress mediated apoptosis in the CF bronchial epithelium. PM causes enhanced activation of mitochondrial signaling pathways via upregulation of the pro-apoptotic mediators Bax, Bad, p53 and p21, and by translocation of Bax to the mitochondria. Further, overexpression of anti-apoptotic Bcl-xL and the inhibition of the mitochondrial respiratory chain block these effects suggesting a critical role of oxidative stress mediated enhanced mitochondrial signaling in PM-induced apoptosis in CF cells. To determine the effects of air pollution in CF, we used IB3-1 human bronchial epithelial (HBE) cells that express a delta F508 and a W1282X non-sense CFTR mutation and compared the effects with the S9 HBE cells which stably express wild-type CFTR and the normal HBE Cells. The S9 cells are derived from the IB3-1 cell line with the CF phenotype corrected by transfection with wild-type adeno-associated viral CFTR.

Our data show that an exposure of IB3-1 cells to PM (25 $\mu g/cm^2$) for 1 h causes a 6.5-fold increase in apoptosis as assessed by DNA Nucleosomal Fragmentation ELISA as compared to 1.7-fold increase in control IB3-1 cells (Fig. 1a). While, PM induces a 3.5-fold increase in apoptosis in S9 cells and in normal HBE cells as compare to their respective controls (Fig. 1a). The IB3-1 CF cell controls show slightly higher but statistically significant (1.7-fold) apoptosis as compared to S9 cell controls, suggesting that CF cells have higher baseline oxidative stress.

Furthermore, IB3-1 CF cells were found to be highly sensitive to PM-induced oxidative stress and apoptosis at much lower dose (25 $\mu g/cm^2$), as compared to previously reported PM-induced apoptosis in alveolar epithelial cells (AEC) and A549 cells at the dose of 100 $\mu g/cm^2$ [6,17]. Therefore, a dose of 25 $\mu g/cm^2$ was used to perform all further experiments in IB3-1 cells. As shown in Fig. 1b–e, PM induces significant decrease in mitochondrial membrane potential and enhances activation of caspases 9 and 3 in IB3-1 cells as compare to S9 cells, followed by activation of Poly (ADP-ribose) polymerase-1 (PARP-1), a modulator of mitochondrial function that rapidly triggers apoptosis by impairing the mitochondrial ATP production in CF cells [17]. These data suggest that

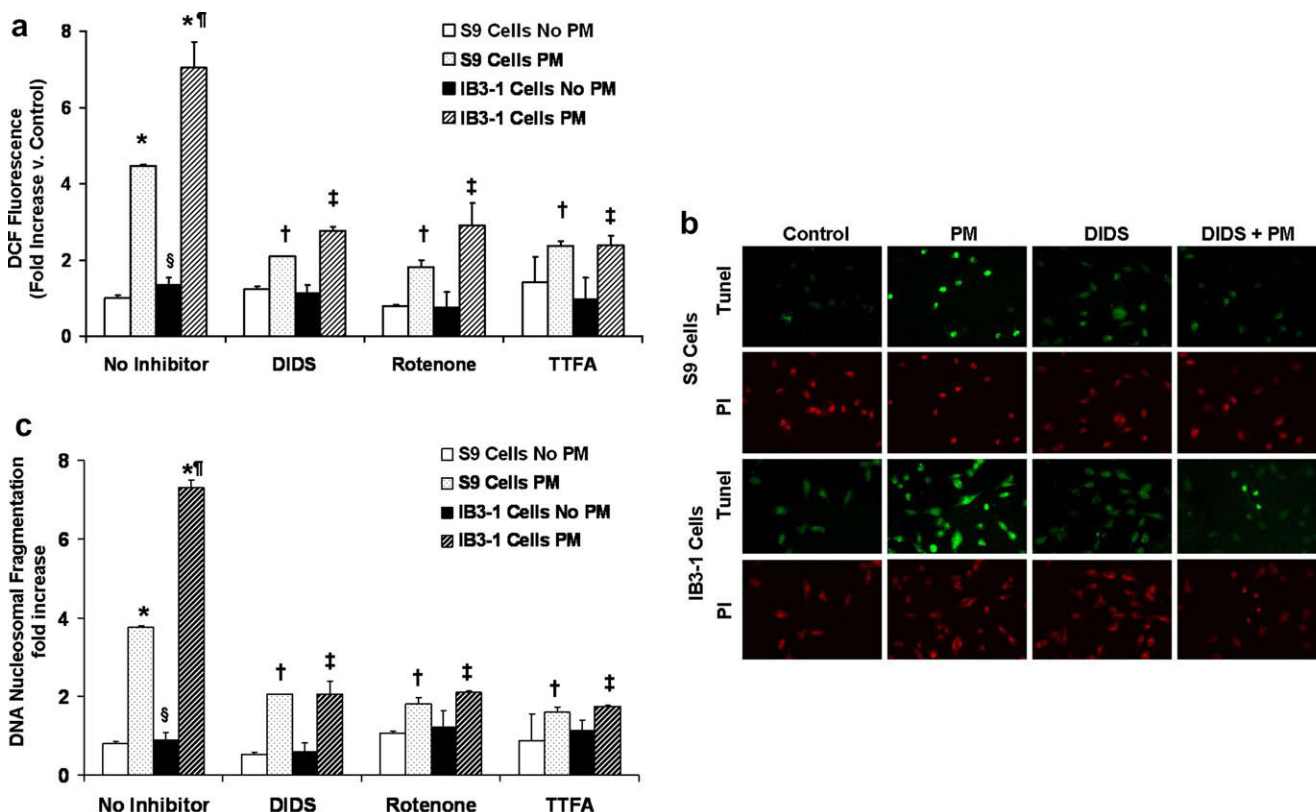

Fig. 2. ROS mediated mitochondrial signaling in CF epithelium: PM enhanced ROS generation in IB3-1 cells as compared to S9 cells; while, mitochondrial inhibitors, DIDS, rotenone and TTFA blocked PM-induced ROS and apoptosis as assessed by TUNEL and DNA fragmentation ELISA (Fig. 2). $^*P < 0.05$ control vs. PM; † and $^\ddagger P < 0.05$ PM vs. inhibitor + PM, $^\P P < 0.05$ S9 cells vs. IB3-1 cells PM, $^\S P < 0.05$ S9 cells control vs. IB3-1 cells control and $n = 3$.

PM-induces enhanced activation of mitochondrial signaling pathway in CF. Increase in the baseline apoptosis in the IB3-1 CF control cells (Fig. 1a) may be as a result of pro-inflammatory signals elicited in these cells due to the increased oxidative stress that may contribute to lung injury and apoptosis. Several inflammatory mediators are involved in this process, in particular, tumor necrosis factor-alpha (TNF-α)-induced generation of mitochondrial ROS and apoptosis has been shown to play a role in CF lung inflammation [18].

Generation of ROS appears to be a central mechanism of PM induced cytotoxicity in various cells [17–20]. Similarly, we found that PM induces generation of ROS in human CF bronchial epithelial cells, however, these effects were seen to be significantly enhanced in IB3-1 CF cells by fivefold as

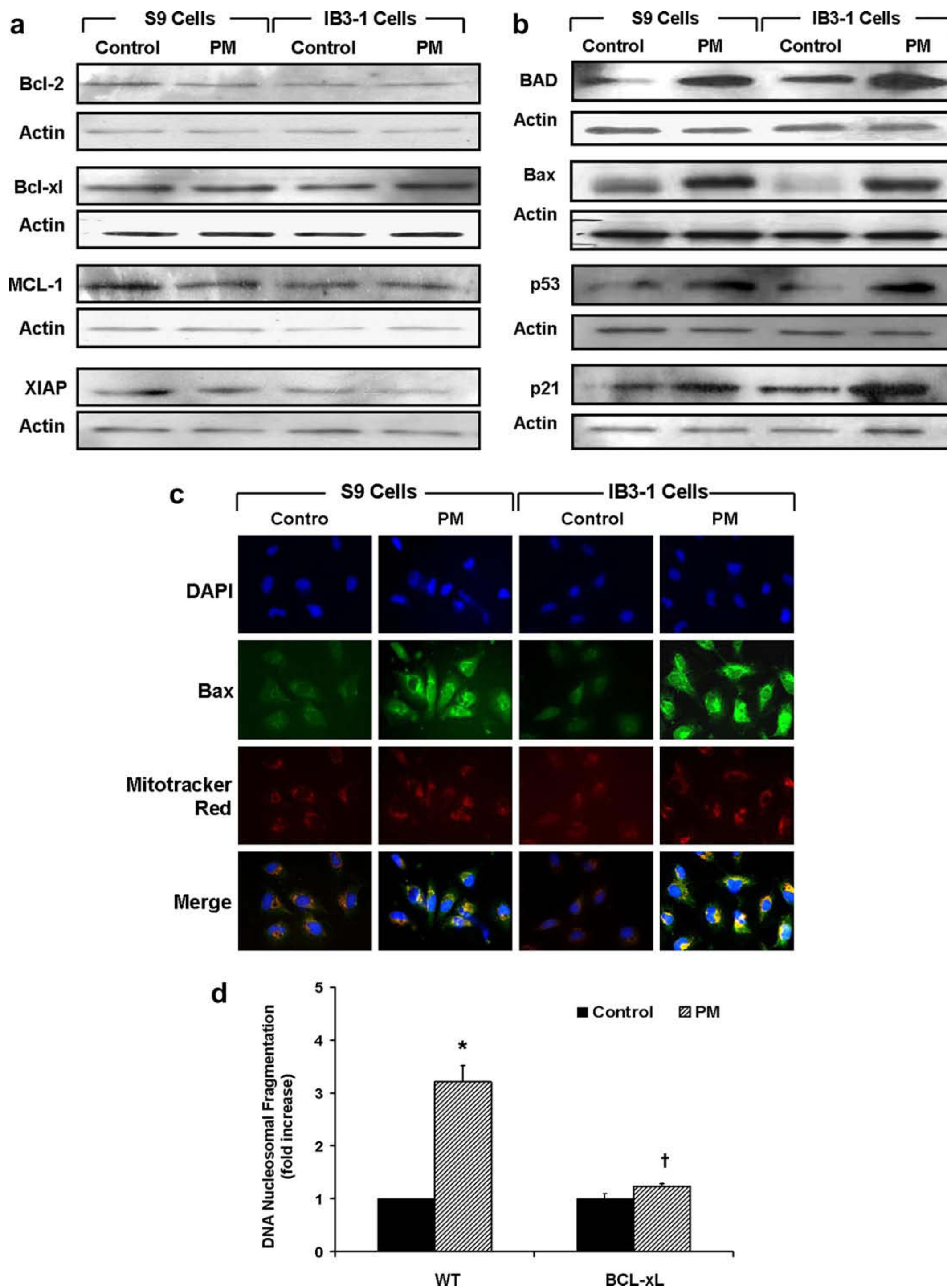

Fig. 3. Pro-apoptotic and anti-apoptotic Bcl regulated mitochondrial signaling mediates PM-induced oxidative stress and apoptosis in CF: PM upregulated pro-apoptotic proteins Bcl2-antagonist of cell death (BAD), Bax, p53 and p21; and enhanced mitochondrial localization of Bax; while, anti-apoptotic Bcl-2, Bcl-xl, Mcl-1 and Xiap were unchanged (Fig. 3a–c). Over expression of anti-apoptotic Bcl-xl inhibited PM-induced apoptosis (Fig. 3d). $*P < 0.05$ control vs. PM; $^{\dagger}P < 0.05$ WT vs. Bcl-xl and $n = 3$.

compared to control IB3-1 cells; while, the S9 cells exposed to PM showed 4.4-fold increase as compared to their controls as assessed by DCF fluorescence assay (Fig. 2a). In addition, as shown in Fig. 2c, PM induced significantly higher, about sevenfold increase in IB3-1 CF cells apoptosis, while 3.7-fold in S9 cells as compared to their respective controls suggesting that the CF cells may exhibit increased susceptibility to oxidative stress and apoptosis.

Mitochondria are the most important intracellular source of ROS. It is also known that the mitochondrial electron transport chain generates ROS in vivo [19–21]. Mitochondrial ROS are transported to the cytoplasm by voltage-dependent anion channels [20]. The mitochondrial anion channel inhibitor, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) protects cells by blocking the egress of ROS from the mitochondria and blocks the response to oxidative stress [20,21]. Further, disruption of mitochondrial electron transport can augment ROS production, which further can amplify apoptotic stimuli. Inhibition of the mitochondrial electron transport chains prevents mitochondrial ATP production by blocking the electron transport thereby attenuate oxidative stress-induced signaling and apoptosis. In this study, we found that DIDS, a mitochondrial anion channel inhibitor; rotenone, an inhibitor of the mitochondrial electron transport chain complex I and thenoyltrifluoroacetone (TTFA), an inhibitor of the mitochondrial electron transport chain complex II block the release of mitochondrial ROS as assessed by DCF fluorescence assay and prevent apoptosis by TUNEL and DNA Fragmentation ELISA (Fig. 2a–c).

Apoptotic stimuli are modulated by the Bcl family of anti-apoptotic proteins (Bcl-2, Bcl-XL and Mcl-1) that prevent apoptosis by inhibiting the reduction in the mitochondrial membrane potential and subsequent activation of caspase 9 and mitochondrial death pathway [10–13]. The inhibitor of apoptosis protein (XIAP) functions through direct interactions to inhibit the activity of caspases 3, 7 and 9 [10–16]. While, the pro-apoptotic Bcl family proteins (e.g., Bad, Bax, Bak, Bim and Bid) translocate from the cytosol to the mitochondrial membrane to induce cell death [10–17]. Bax is a key component for cellular induced apoptosis through mitochondrial stress [13,16]. Moreover, the pro-apoptotic family member, such as, Bad is known to promote cell death by displacing Bax from binding to Bcl-2 and Bcl-xL [10–13,17]. As shown in Fig. 3a and b, PM upregulates pro-apoptotic proteins Bad, Bax, p53 and p21 in CF cells, while, the levels of the anti-apoptotic proteins Bcl-2, Bcl-XL, Mcl-1 and XIAP remain unchanged.

The migration and mitochondrial translocation of Bax in response to oxidative stress increases the mitochondrial membrane's permeability through interactions with pore proteins on the mitochondrial membrane which leads to activation of caspases and apoptosis [13,16]. Using immunocytochemical staining with the mitochondrial marker, mitotracker red, in this study, we examined the effects of PM in CF cells. As shown in Fig. 3c, exposure to PM causes induction, translocation and co-localization Bax to the mitochondria in IB3-1 and S9 cells leading to apoptosis; this effect was seen to be more pronounced in IB3-1 cells as compared to S9 cells. Furthermore, overexpression of anti-apoptotic Bcl-xl blocks PM-induced apoptosis in epithelial cells (Fig. 3d). Collectively, these data show that upregulation of pro-apoptotic mediators and mitochondrial translocation of Bax plays a crucial role in modulating PM-induced apoptosis in CF bronchial epithelial cells. Overexpression of anti-apoptotic Bcl-xl inhibits PM-induced apoptosis suggesting that Bcl mediated mitochondrial signaling is critically important in PM-induced apoptosis. A hypothetical model based on these results is shown in Fig. 4.

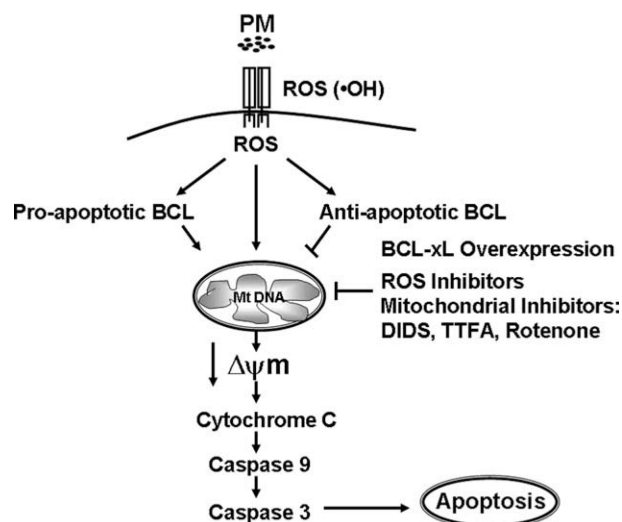

Fig. 4. Schematic diagram of Bcl mediated mitochondrial signaling by PM in human cystic fibrosis bronchial epithelium.

In summary, we demonstrate that PM induces enhanced oxidative stress by the generation of mitochondrial ROS leading to pro-apoptotic and anti-apoptotic Bcl family mediated mitochondria-regulated oxidative stress and apoptosis in CF. These findings suggest that the airway epithelium in inflammatory airway diseases, such as, CF, undergo an intense response to the oxidative stress induced by air pollution; and an impairment of mitochondrial function by the PM plays a critical role in inducing cytotoxic effects of PM. Reduction of oxidative stress in CF may limit the toxic effects of air pollution to the lung.

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