Airborne Particulate Matter Inhibits Alveolar Fluid Reabsorption in Mice via Oxidant Generation

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Ambient particulate matter is increasingly recognized as a significant contributor to human cardiopulmonary morbidity and mortality in the United States and worldwide. We sought to determine whether exposure to ambient particulate matter would alter alveolar fluid clearance in mice. Mice were exposed to a range of doses of a well-characterized particulate matter collected from the ambient air in Düsseldorf, Germany through a single intratracheal instillation, and alveolar fluid clearance and measurements of lung injury were made. Exposure to even very low doses of particulate matter (10 μg) resulted in a significant reduction in alveolar fluid clearance that was maximal 24 h after the exposure, with complete resolution after 7 d. This was paralleled by a decrease in lung Na,K-ATPase activity. To investigate the mechanism of this effect, we measured plasma membrane Na,K-ATPase abundance in A549 cells and Na,K-ATPase activity in primary rat alveolar type II cells after exposure to particulate matter in the presence or abscence of the combined superoxide dismutase and catalase mimetic EUK-134 (5 μM). Membrane but not total protein abundance of the Na,K-ATPase was decreased after exposure to particulate matter, as was Na,K-ATPase activity. This decrease was prevented by the combined superoxide dismutase/catalase mimetic EUK-134. The intratracheal instillation of particulate matter results in alveolar epithelial injury and decreased alveolar fluid clearance, conceivably due to downregulation of the Na,K-ATPase.

Keywords: antioxidant; lung injury; Na,K-ATPase; pollution; ROS

Ambient particulate matter (PM) is increasingly recognized as a significant contributor to human cardiopulmonary morbidity and mortality both in the United States and worldwide (1, 2). There are strong epidemiologic data that link the daily levels of ambient PM to pulmonary symptoms, rates of infections, school absenteeism, daily use of specific medications, hospitalizations for cardiopulmonary disease, and daily cardiopulmonary mortality rates (1–4). Longer term exposure to ambient PM has been associated with an increase in the prevalence of obstructive lung disease and the development of lung cancer (1, 2). Recent estimates suggest that the average loss of life expectancy related to chronic air pollution exposure is between 1.8 and 3.1 yr for individuals living in the most polluted American cities (5).

The precise mechanisms by which exposure to PM contributes to cardiopulmonary mortality are unknown. It is hypothesized that exposure of epithelial cells and macrophages to ambient PM within the respiratory tract results in the development of reactive oxygen species (ROS) that in turn induce injury and inflammation (6). Direct injury to the alveolar epithelium may induce some of the pulmonary complications of exposure to PM, while the resulting inflammatory changes may induce effects in other organs, particularly the cardiovascular system (5). We have observed that exposure to a well-characterized PM collected from ambient air in Düsseldorf, Germany results in oxidant-mediated injury of alveolar epithelial cells (7). We sought to test the hypothesis that exposure of mice to concentrations of PM similar to those found in the ambient air would result in an oxidant-mediated decrease in the protein abundance of the Na,K-ATPase at the plasma membrane of alveolar epithelial cells and decreased edema clearance. We found that the intratracheal administration of ambient PM resulted in decreased clearance of alveolar edema fluid in mice and decreased Na,K-ATPase activity *in vivo* and *in vitro*. *In vitro*, this effect was attenuated by the administration of an antioxidant.

MATERIALS AND METHODS

Animals and Exposure of Mice to Particulate Matter

The protocol for the use of mice was approved by the Animal Care and Use Committee at Northwestern University. Six- to eight-week-old C57BL/6 mice, 20–25 g, were anesthetized with pentobarbital (50 mg/kg) and a 20-gauge angiocath was placed transorally into the trachea under direct visualization. We instilled either PM suspended in 50 μl of sterile PBS or 50 μl of sterile PBS in two equal aliquots, 3 min apart. PM was vortexed before instillation. After each aliquot the mice were placed in the right and then the left lateral decubitus position for 10 s each. Bronchoalveolar lavage fluid was obtained by three sequential instillations and aspirations of 1 ml of sterile cold PBS through a tracheostomy tube. Histology was performed by inflating the lungs with cold PBS to a pressure of 25 cm H_2O, heating them to 60°C and then inflating them with 4% paraformaldehyde. After 16 h the lungs were removed from paraformaldehyde and placed in PBS.

Particulate Matter

The particulate matter was collected by baghouse from ambient air in Düsseldorf, Germany. The particle sample was aerosolized from a turntable into a small-scale powder disperser using 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 and the aerosol was sampled on four occasions for 20 s. The particulate matter < 10 μM in diameter (PM_{10}) was used in these studies. The characteristics of the particulate matter have been previously reported (7). Endotoxin was not detectable in the particulate matter as measured by the E-toxin kit (Sigma-Aldrich, St. Louis, MO). Additional information on the characteristics of the particulate matter and its collection is provided in the online supplement (Table E1).

Measurement of Edema Clearance in Mice

The method used to quantify the rate of removal of fluid from the alveolar airspace (alveolar fluid clearance) was as previously described except that mice were maintained supine (8, 9). Alveolar fluid clearance was calculated based on the change in concentration of Evan's blue–tagged

(*Received in original form August 29, 2005 and in final form December 29, 2005*)

This work was supported by HL071643, HL067835, HL48129, HL076139, the American Heart Association, the American Lung Association, and the American Lung Association of Metropolitan Chicago.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 34. pp 670–676, 2006
Originally Published in Press as DOI: 10.1165/rcmb.2005-0329OC on January 26, 2006
Internet address: www.atsjournals.org

isotonic, iso-osmolar albumin solution instilled into the alveolar airspace over a 30-min period of measurement.

Cell Culture

Primary rat alveolar type II epithelial cells were isolated as previously described and were used on Day 2 after isolation (10). Both primary rat alveolar type II cells and human alveolar epithelial cells (A549) were cultured in serum-free Dulbecco's modified essential medium supplemented with penicillin (200 U/ml) and streptomycin (200 μg/ml), 20 mM HEPES, and 10% heat-inactivated fetal calf serum (37°C, 5% CO_2) (all from Invitrogen, Carlsbad, CA). The A549 cells used in these experiments stably expressed a GFP-labeled α1 subunit of the Na,K-ATPase (a kind gift of Dr. Alejandro Bertorello [Karolinska Institute, Stockholm, Sweden] [11]). Cells were exposed to PM diluted in PBS or vehicle. To ensure equal dispersion, the PM solution was vortexed with the media before application to the cells.

Cell Surface Labeling and Immunoblotting

Cells were labeled for 1 h using 0.5 mg/ml EZ-link NHS-SS-biotin (Pierce Chemical Co., Rockford, IL). After labeling, the cells were rinsed three times with PBS containing 50 mM glycine to quench unreacted biotin and then lysed in modified radioimmunoprecipitation buffer (mRIPA; 50 mM Tris-HCl [pH 8], 150 mM NaCl, 1% NP-40, and 1% sodium deoxycholate, containing protease inhibitors as described above). Aliquots (75 μg of protein) were incubated overnight at 4°C with end-over-end shaking in the presence of streptavidin beads (Pierce Chemical Co.). The beads were thoroughly washed and then resuspended in 30 μl of Laemmli sample buffer solution. Proteins were analyzed by SDS-PAGE and Western blot.

Assessment of Basolateral Na,K-ATPase Activity in Membrane Proteins

Whole cell membrane proteins were obtained peripheral lung tissue that was homogenized in (mmol/liter): 300 mannitol, 10 Hepes-Tris (pH 7.4) with 3 EGTA/1 EDTA, 0.1 PMSF, and 0.01 mg/ml N-tosyl-L-phenylalanine chlorylmethyl ketone, 0.01 mg/ml leupeptin (homogenization buffer; all from Sigma). Cell debris was removed by centrifugation at 10,000 × *g* for 20 min at 4°C. The resultant supernatant was twice centrifuged at 100,000 × *g* at 4°C, and the resultant pellets resuspended in 100 μl of homogenization buffer. Twenty micrograms of BLM protein was resuspended in 100 μl of a high $[Na^+]$/low $[K^+]$ reaction buffer (in mmol/liter: 50 Tris-HCl pH 7.4, 50 NaCl, 5 KCl, 10 Mg_2Cl, 1 EGTA, 10 Na_2ATP, with [γ-^{32}P]–ATP [3.3 nCi/μl]). Triplicate samples were placed at −20°C for 15 min before incubation for 15 min at 37°C. The reaction was terminated by addition of 5% TCA/10% charcoal and cooling to 4°C. The charcoal phase containing unhydrolyzed nucleotide was separated by centrifugation (12 000 × *g* for 5 min) and the liberated ^{32}P quantified. Na,K-ATPase activity was calculated as the difference between the test samples (total ATPase activity) and samples assayed in reaction buffer with 2.5 mmol/liter ouabain but devoid of Na^+ and K^+ (nonspecific ATPase activity). Results are expressed as nmol of P_i/mg of protein/h (9).

Measurement of Na,K-ATPase Activity *In Vitro*

Na,K-ATPase activity was determined as the rate of [^{32}P]-ATP hydrolysis. The ouabain-insensitive ATPase activity was measured in buffer lacking Na^+ and K^+ but including 5 mM ouabain. Nonspecific ATP hydrolysis was measured in the absence of cells. The [^{32}Pi] liberated was quantitated by scintillation counting and expressed as μmol P_i/mg protein/h (12).

Calculation of the Lung Injury Score

The lung injury score was calculated using the method described by Matute-Bello and colleagues (13). Blinded lung sections stained with hematoxylin and eosin were scored using a 0–3 scale by an investigator for the presence of interstitial and alveolar inflammation, alveolar hemorrhage, and edema where normal was scored as 0, 1 as mild, 2 as moderate, and 3 as severe. The resulting three scores were averaged and presented as the lung injury score for that section. Two sections per animal were scored independently.

Measurement of Reduced Glutathione

Reduced glutathione was measured using a commercially available assay (ApoGSH glutathione detection kit; BioVision, Inc., Mountain View, CA). Plates were removed from experimental conditions and placed immediately on ice. Medium was aspirated and cells were washed with ice-cold PBS. Each plate was incubated on ice with cell lysis buffer (10 min). Cells were scraped using a cell scraper. The lysate was collected, homogenized (30 s), and centrifuged (10 min). The glutathione level was expressed as OD per microgram of protein (Bradford) (10).

Statistics

ANOVA was used to test for significant differences in measured variables between groups. Where the F statistic indicated a significant difference, individual differences were explored using the Bonferroni correction for multiple comparisons. Statistical significance was determined at the 0.05 level.

RESULTS

Exposure to Airborne PM Reduces Alveolar Fluid Clearance in Mice

We hypothesized that exposure of mice to airborne PM might result in epithelial dysfunction characterized by a decrease in the rate of edema clearance. To test this hypothesis, airborne PM was suspended in PBS and instilled into the lungs of mice through a 20-gauge angiocath placed into the trachea under direct visualization. Exposure to PM resulted in a dose-dependent reduction in alveolar fluid clearance that was significantly different from saline-treated controls starting at doses of 10 μg or more (Figure 1A). At a dose of 100 μg, instillation of PM resulted in decreased clearance of fluid that was maximal after 24 h and recovered by 7 d after the exposure (Figure 1B). These results suggest that the intratracheal instillation of PM results in dose-dependent decreases in alveolar fluid clearance that are maximal after 24 h and, without further PM exposure, resolve within 7 d.

Exposure to PM Results in Mild Lung Injury after 24 h

To determine whether the decrease in alveolar fluid clearance we observed after exposure to PM was a consequence of lung

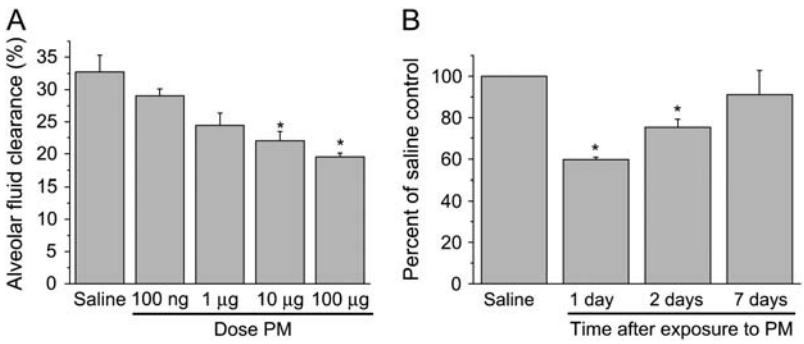

Figure 1. The intratracheal instillation of PM reduces alveolar fluid clearance in mice. Anesthetized mice were intratracheally intubated and PM was instilled into the lungs at the doses indicated. (*A*) Alveolar fluid clearance was measured 24 h after the instillation of PM or saline. (*B*) Mice were administered 100 μg of PM or saline intratracheally, and alveolar fluid clearance was measured at the times indicated. *$P < 0.05$; all observations represent three or more animals.

injury, we treated animals with PM at varying doses and examined hematoxylin and eosin–stained lung sections from animals killed 24 h later. A lung injury score was calculated by a blinded review of the histologic sections where a normal lung received a score of 0, and mild, moderate, or severe lung injury received scores of 1, 2, and 3, respectively. Lungs from mice treated with intratracheal LPS (6 I.U./kg) served as in internal control. Histologic evidence of lung injury was not evident at doses of PM $<$ 100 μg (Figure 2A). At doses of PM of 100 μg or more, there was evidence of mild lung injury and pleural reaction (Figures 2A and 2B). To determine whether exposure to PM resulted in delayed evidence of lung injury, we examined lung sections 7 d after exposure to PM (10 μg). The lungs were indistinguishable from saline-treated lungs at this time point

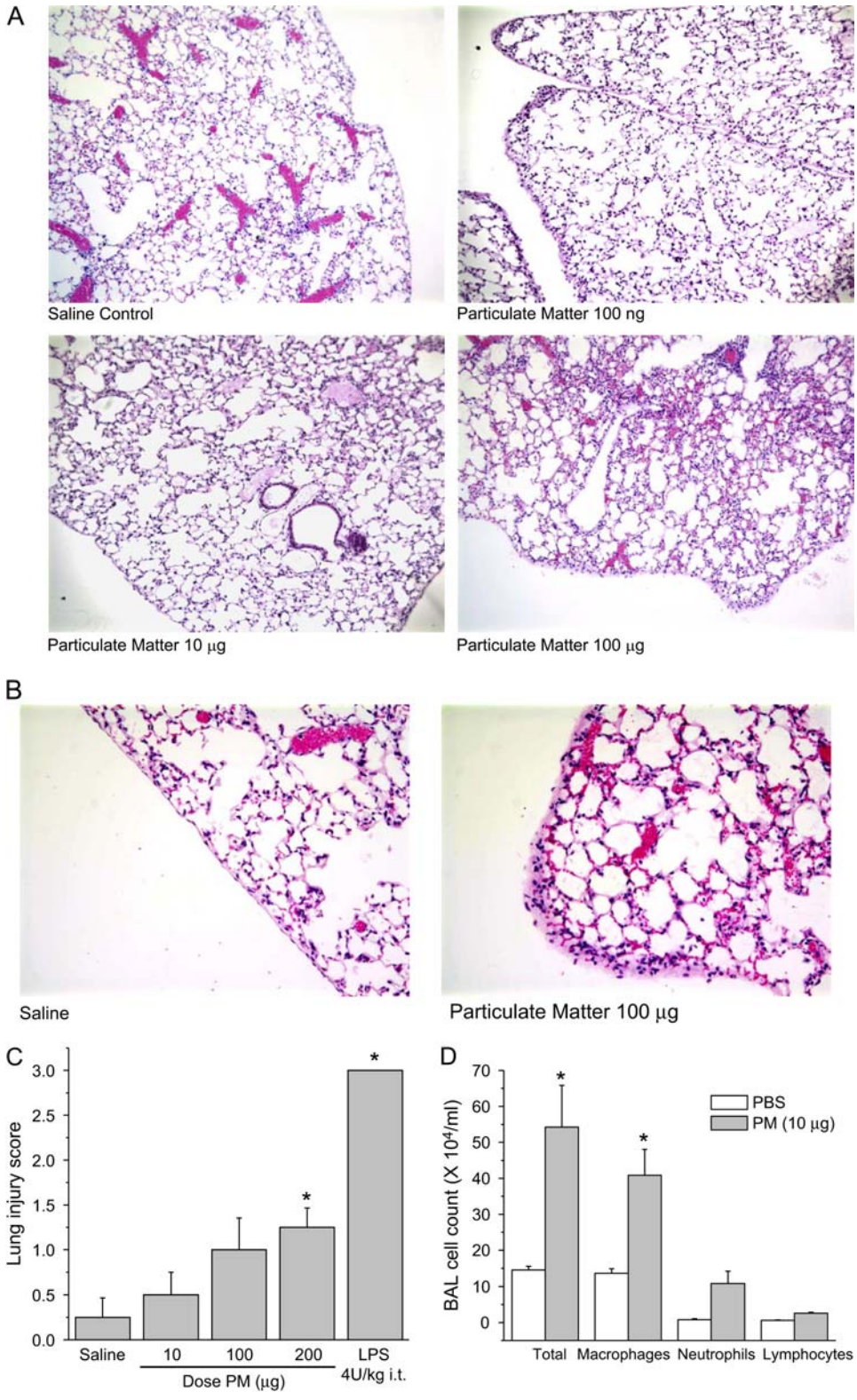

Figure 2. Exposure to PM results in minimal lung injury. Mice were administered PM at the doses indicated, and 24 h later the lungs were harvested for histologic examination. (*A*) Representative low-power (×200) views of the lungs of mice treated with increasing doses of PM. (*B*) Higher-power view (×400) of the lungs of mice 24 h after treatment with saline or PM (100 μg) showing mild interstitial mononuclear inflammation and pleural reaction. (*C*) Blinded pathologic scoring of lung injury 24 h after the intratracheal instillation of saline or PM (0 = normal, 1 = mild, 2 = moderate, 3 = severe lung injury). (*D*) Cell counts and differentials in the bronchoalveolar lavage fluid obtained from animals treated with PBS or PM. *$P < 0.05$ for comparison between PBS- and PM-treated animals. All observations represent the findings from four or more animals.

(data not shown). Bronchoalveolar lavage fluid from the lungs of animals was collected 24 h after PM exposure for cell count and differential. Compared with PBS-treated animals, animals exposed to PM demonstrated a significant increase in BAL fluid total cells ($14.5 \pm 1.0 \times 10^4$ versus $54.3 \pm 11.6 \times 10^4$ cells/ml) and alveolar macrophages ($13.6 \pm 1.3 \times 10^4$ versus $40.8 \pm 7.2 \times 10^4$ cells/ml). Neutrophils were also increased 0.8 ± 0.2 versus 10.8 ± 3.3 cells/ml), but this did not reach statistical significance ($P = 0.07$). Wet-to-dry ratios were not increased from baseline at any dose of PM (data not shown). These results suggest that the reductions in alveolar fluid clearance observed after the instillation of PM are not associated with significant lung injury or the development of pulmonary edema.

Na,K-ATPase Activity Is Inhibited in Basolateral Membranes of Peripheral Lung Tissue after Exposure to PM

In other models of lung injury, for example hyperoxia, we have reported that decreased alveolar fluid clearance is associated with decreased activity of the Na,K-ATPase in the basolateral membrane of alveolar epithelial cells (14). To determine whether the reduction in alveolar fluid clearance we observed after the instillation of PM was associated with a reduction in Na,K-ATPase activity *in vivo*, we instilled PM (100 μg) or saline into the lungs of mice and isolated basolateral membranes from peripheral lung tissue 24 h later. A separate set of animals was treated with 50 μl of 10^{-4} M isoproterenol for 30 min before harvest of the lungs as a positive control. The Na,K-ATPase activity in basolateral membranes isolated from PM-treated mice was significantly reduced compared with that in saline-treated mice (Figure 3). These results suggest that decreased expression of the Na,K-ATPase in the basolateral membrane of alveolar epithelial cells might account for the reduction in edema clearance observed after treatment with PM.

PM Decreases the Na,K-ATPase Plasma Membrane Protein Abundance in Alveolar Epithelial Cells

The decrease in Na,K-ATPase activity in the basolateral membranes isolated from peripheral lung tissue in PM-exposed mice might result from a direct effect of PM on the alveolar epithelium or might result from the activation of macrophages or other inflammatory cells with secondary effects on the alveolar epithelium. To determine whether direct exposure to PM might result in a decrease in the plasma membrane protein abundance of the Na,K-ATPase in alveolar epithelial cells, we exposed A549 cells constitutively expressing a green fluorescent protein (GFP)-labeled α1 subunit of the Na,K-ATPase to PM and measured membrane abundance of the Na,K-ATPase using a membrane pulldown assay. Exposure to PM resulted in a decrease in plasma membrane Na,K-ATPase protein abundance in the plasma membrane (Figure 4A). Total Na,K-ATPase levels were unchanged after exposure to PM (Figure 4B). These data suggest that endocytosis of the Na,K-ATPase from the basolateral plasma membrane is a potential mechanism by which exposure to PM might inhibit alveolar fluid clearance.

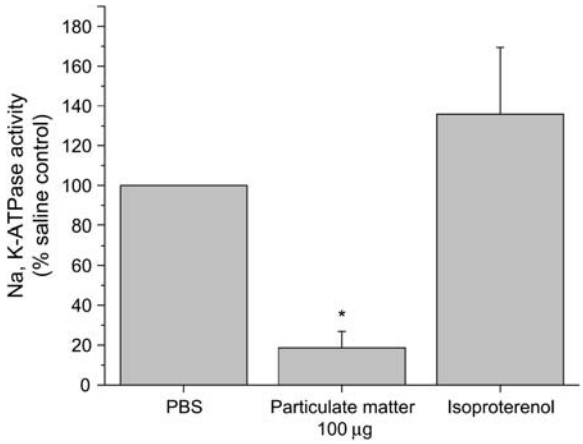

Figure 3. Basolateral Na,K-ATPase activity after exposure to PM. PM (10 μg/animal) or saline was delivered intratracheally to mice, and 24 h later the lung tissue was harvested and the membrane fraction was isolated. A separate set of animals was treated with 50 μl of 10^{-4} M isoproterenol (30 min) as a positive control. Na,K-ATPase activity was measured by the ouabain-sensitive liberation of ^{32}P from $AT^{32}P$. *$P <$ 0.05 compared with saline controls; each observation represents the average of three or more experiments.

The PM-Induced Decrease in Plasma Membrane Na,K-ATPase Abundance Requires the Generation of ROS

Exposure to PM has been shown to result in increased generation of ROS, particularly by the mitochondria. To determine whether oxidant generation was responsible for the decrease in Na,K-ATPase abundance in the plasma membrane after exposure to PM, we treated A549 cells constitutively expressing a GFP-labeled α1 subunit of the Na,K-ATPase with the antioxidant EUK-134 and exposed them to PM for 24 h. EUK-134 is a combined superoxide dismutase and catalase mimetic that has been shown to prolong the lifespan of the nematode *Caenorhabditis elegans* and to prevent alveolar epithelial cell death during hyperoxia (10, 15). Treatment with EUK-134 prevented the reduction in the levels of reduced glutathione observed after exposure to PM Figure 5A. Cells were then exposed to PM in the presence or absence of EUK-134 (5 μM), and protein abundance of the Na,K-ATPase at the alveolar epithelial cell basolateral membrane was measured 24 h later. Treatment with EUK-134 attenuated the decrease in the plasma membrane protein abundance of the Na, K-ATPase after exposure to PM (Figure 5B). These results suggest that the generation of ROS is required for the decrease in membrane expression of the Na,K-ATPase induced by PM.

Exposure to PM Results in Decreased Na,K-ATPase Activity in Primary Rat Alveolar Type II Cells that Requires the Generation of ROS

Reductions in membrane abundance of the Na,K-ATPase are usually paralleled by reductions in ouabain-sensitive Na,K-ATPase activity. Consistent with this observation, we found that treatment with PM resulted in dose-dependent reductions in the ouabain-sensitive liberation of ^{32}P from labeled $AT^{32}P$ in primary rat alveolar type II cells. The reduction in Na,K-ATPase activity was abolished in cells treated with EUK-134 (20 μM) (Figure 6).

DISCUSSION

The toxic effects of air pollution are thought to be associated predominantly with exposures to ozone, nitrogen dioxide, sulfur dioxide, and PM (reviewed in Ref. 1). Particulate matter is that mixture of solid and liquid particles suspended in the air. It is a temporally and spatially shifting combination originating from both natural and anthropogenic sources (reviewed in Ref. 1). The particles range from a few nanometers to several millimeters. Short-term exposure to PM has been associated with short-term increases in mortality as well as morbidity from cardiopulmonary disease (2, 16). In the 90 largest cities in the United States, investigators conducting the National Mortality, Morbidity and Air Pollution Studies (NMMAPS) demonstrated an increase

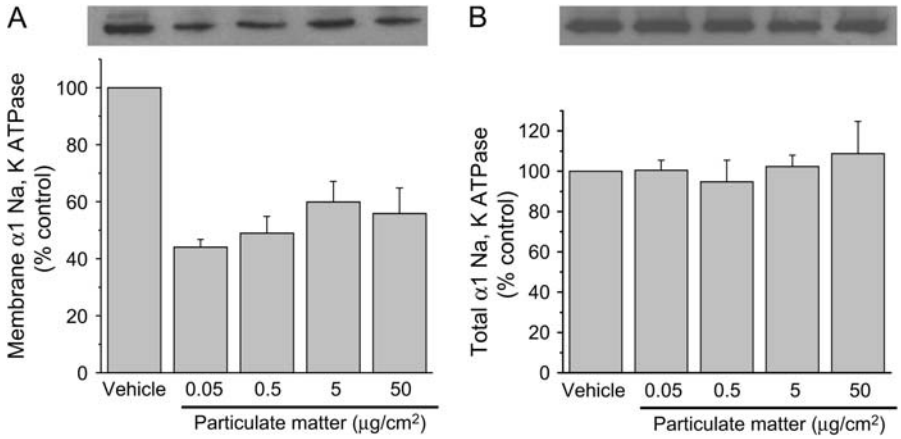

Figure 4. Administration of PM results in a reduction in the membrane abundance of the Na,K-ATPase in alveolar epithelial cells. (*A*) Alveolar epithelial cells (A549) constitutively overexpressing a GFP-labeled α_1 subunit of the Na,K-ATPase were treated with vehicle or PM and membranes were biotin-labeled and immunoprecipitated. The resulting proteins were immunoblotted using an antibody against GFP. (*B*) Whole cell lysates were obtained from A549 cells treated with PM and immunoblotted with an antibody against GFP. $*P < 0.05$ for comparison with the untreated control. Each observation represents the average of three or more experiments.

in all-cause daily mortality of 0.21% for every 10 $\mu g/m^3$ increase in the PM_{10} (17, 18). In 29 European cities, investigators conducting the Air Pollution and Health: a European Approach (APHEA-2) study found similar associations between daily PM_{10} levels and short-term mortality (0.6% per 10 $\mu g/m^3$) (19). Positive associations have also been found between daily levels of PM_{10} and hospital admissions, particularly for pulmonary and cardiac diseases.

We found that exposure to well-characterized PM collected from the air of an industrial city resulted in dose-dependent inhibition of the ability of the lung to clear edema fluid. These findings were paralleled by a decrease in the activity of the Na,K-ATPase measured in basolateral membrane fractions isolated from the intact lung, suggesting that the observed reduction in alveolar fluid clearance after exposure to PM might result from a reduction in active Na^+ transport. Measurement of Na,K-ATPase activity in membrane fractions from whole lung homogenates might reflect alterations of Na,K-ATPase activity in multiple cells within the lung; however, the majority of the resistance to solute transport in the lung is provided by the alveolar epithelium (20). Experiments in alveolar epithelial cells *in vitro* showed decreased Na,K-ATPase protein abundance at the plasma membrane and decreased Na,K-ATPase activity. The finding that inhibition of PM-induced oxidant production prevents the decrease in Na,K-ATPase plasma membrane protein abundance and Na,K-ATPase activity suggests that ROS generated in response to PM might cause a reduction of alveolar fluid clearance by promoting endocytosis of the Na,K-ATPase from the basolateral membrane into intracellular pools.

In our study, PM were administered using a single intratracheal instillation. This likely results in a different distribution of PM than would be obtained if the particulates were inhaled. For example, Crapo and colleagues performed elegant microdissection experiments that showed asbestos fibers delivered by aerosolization to rats were concentrated at branch points in the proximal small airways, the same regions in which lung injury was most severe (21). In the lungs of patients from Vancouver, Churg and Brauer found a 25- to 100-fold increase in particle deposition in the respiratory bronchioles compared with other tissues (22). This inequality of distribution is more pronounced in individuals with obstructive lung disease that exhibit regional heterogeneity of ventilation (6). The distribution of inhaled particles in the airways also varies as a function of particle size; larger particles are distributed primarily in the nasopharynx and

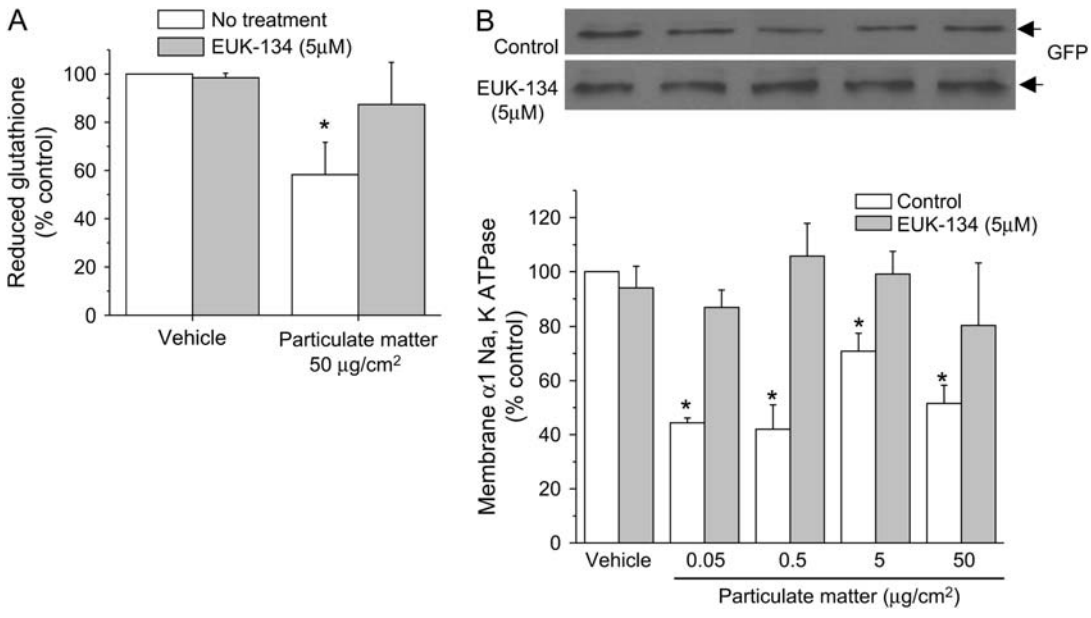

Figure 5. Antioxidants prevent the PM-induced reduction in membrane abundance of the Na,K-ATPase in alveolar epithelial cells. (*A*) Alveolar epithelial cells were exposed to PM or to vehicle in the presence or absence of EUK-134 (5 μM) for 24 h, and the level of reduced glutathione in the whole cell lysate was measured. (*B*) Alveolar epithelial cells were treated with PM in the presence or absence of EUK-134 (5 μM) and membrane abundance of the Na,K-ATPase was measured. $*P <$ 0.05 for comparison with the untreated control. Each observation represents the average of three or more experiments.

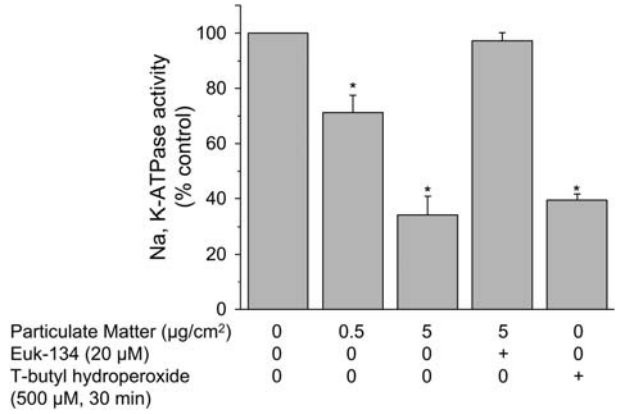

Figure 6. Exposure to PM results in decreased activity of the Na,K-ATPase in primary rat alveolar type II cells. Two days after isolation, cells were treated with PM in the presence or absence of EUK-134 (20 μM) and Na,K-ATPase activity was measured as the ouabain sensitive liberation of ^{32}P from $AT^{32}P$. Treatment with t-butyl hydroperoxide (500 μM, 30 min) was used as a positive control. $*P < 0.05$ for comparison with control. Each observation represents the average of three or more experiments.

proximal airways, while smaller particles reach the small airways and alveolar epithelium (22). This distribution might have important implications for toxicity, as the smallest particles appear to account for much of the morbidity and mortality attributable to PM (5).

Our results demonstrating that the reduction in Na,K-ATPase activity and membrane abundance of the Na,K-ATPase in alveolar epithelial cells requires the generation of oxidants is consistent with the findings of other investigators. There is extensive evidence both *in vitro* and *in vivo* showing that exposure to PM results in an increase in the generation of ROS (reviewed in Ref. 6). Nel and colleagues demonstrated that both diesel exhaust particles and methanol extracts of these particles induce ROS production in lung microsomes and isolated mitochondria, suggesting that organic chemicals on the particle were responsible for the ROS production (23). They eluted the chemicals and found that fractions enriched in polycyclic aromatic hydrocarbons (which can be metabolized to quinones), and fractions enriched in quinones, were responsible for oxidant generation (6, 23–27). Based on these data, they suggested that quinones undergoing one-electron-transfer reactions to semiquinones might be responsible for the generation of superoxide radical induced by PM (6). These quinones could donate their electrons to electron acceptors in the electron transport chain in the mitochondria, the NAD(P)H oxidase in the plasma membrane, the P450 oxidase in microsome or other intracellular locations. In the presence of transition metals (e.g., iron contained in the particle) and SOD enzymes, H_2O_2-derived hydroxyl radicals might be generated enhancing the particle's toxicity.

Recently, Arimoto and coworkers reported that exposure of mice to diesel exhaust PM sensitized the animals to LPS-induced lung injury through an ROS-dependent mechanism (28). In that report, the authors found that exposure to diesel PM with LPS resulted in the development of severe lung injury with pronounced pulmonary edema. Our results suggest that PM-induced oxidant-mediated reductions in alveolar fluid clearance may account for part of this sensitizing effect.

Our results are also consistent with our previous report that exposure to PM resulted in oxidant-mediated apoptosis of alveolar epithelial cells (7). In that report, the dose of PM that was required to induce cell death was substantially higher than the dose we observed to cause a decrease in plasma membrane protein abundance of the Na,K-ATPase and a decrease in Na,K-ATPase activity in the lung. This finding might explain our observation that reductions of alveolar fluid clearance in response to the administration of PM occurred with only mild histologic evidence of lung injury.

The doses that resulted in significant effects in our study were similar to those that might be delivered to the respiratory system in many urban environments. For example, in 2002, the U.S. Environmental Protection Agency reported a relatively wide range of maximal city-specific PM_{10} concentrations between 26 and 534 $\mu g/m^3$ (5). Using the highest value and assuming a minute ventilation of 1.7 ml/g/min for a mouse, the total dose inhaled over 24 h would be 25 μg.

We and others have observed that changes in alveolar fluid clearance are paralleled by changes in the plasma membrane protein abundance of the Na,K-ATPase in alveolar epithelial cells (20, 29–32). We recognize, however, that other factors may contribute to the PM-induced reduction in alveolar fluid clearance. For example, downregulation of the epithelial Na^+ channel (ENaC) has been shown to be an important mechanism regulating alveolar fluid clearance (33, 34). A complete characterization of PM-induced changes in alveolar fluid transport is beyond the scope of this report.

The molecular mechanisms that underlie the decrease in Na,K-ATPase activity and membrane abundance of the Na,K-ATPase in alveolar epithelial cells in response to PM are not known. However, other stimuli that cause the endocytosis of the Na,K-ATPase through an oxidant-dependent mechanism, including the exogenous administration of H_2O_2 and hypoxia, require PKC-mediated phosphorylation of α_1-subunit of the Na,K-ATPase (12, 35). We speculate that the generation of ROS induced by exposure to particulate matter might result in PKC-mediated phosphorylation and subsequent endocytosis of the Na,K-ATPase from the basolateral membrane.

In summary, we report that the intratracheal instillation of airborne PM results in a reversible, dose-dependent reduction in alveolar fluid clearance in mice. *In vitro* data support a role for oxidant generation in the mechanism underlying this observation. This decrease in alveolar clearance may affect the ability of the animal to tolerate damage to the alveolar capillary barrier and hydrostatic or oncotic changes that favor the formation of alveolar fluid.

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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