In vivo and in vitro proinflammatory effects of particulate air pollution (PM10)

In Vivo and *in Vitro* Proinflammatory Effects of Particulate Air Pollution (PM_{10})

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Epidemiologic studies have reported associations between fine particulate air pollution, especially particles less than 10 mm in diameter (PM_{10}), and the development of exacerbations of asthma and chronic obstructive pulmonary disease. However, the mechanism is unknown. We tested our hypothesis that PM_{10} induces oxidant stress, causing inflammation and injury to airway epithelium. We assessed the effects of intratracheal instillation of PM_{10} in rat lungs. The influx of inflammatory cells was measured in bronchoalveolar lavage (BAL). Airspace epithelial permeability was assessed as total protein in bronchoalveolar lavage fluid (BALF) *in vivo*. The oxidant properties of PM_{10} were determined by their ability to cause changes in reduced glutathione (GSH) and oxidized glutathione (GSSG). We also compared the effects of PM_{10} with those of fine (CB) and ultrafine (ufCB) carbon black particles. Six hours after intratracheal instillation of PM_{10}, we noted an influx of neutrophils (up to 15% of total BAL cells) in the alveolar space, increased epithelial permeability, an increase in total protein in BALF from 0.39 ± 0.01 to 0.62 ± 0.01 mg/ml (mean ± SEM) and increased lactate dehydrogenase concentrations in BALF. An even greater inflammatory response was observed after intratracheal instillation of ufCB, but not after CB instillation. PM_{10} had oxidant activity *in vivo*, as shown by decreased GSH in BALF (from 0.36 ± 0.05 to 0.25 ± 0.01 nmol/ml) after instillation. BAL leukocytes from rats treated with PM_{10} produced greater amounts of nitric oxide, measured as nitrite (control 3.07 ± 0.33, treated 4.45 ± 0.23 mM/1×10^6 cells) and tumor necrosis factor alpha (control 21.0 ± 3.1, treated 179.2 ± 29.4 unit/1×10^6 cells) in culture than BAL leukocytes obtained from control animals.These studies provide evidence that PM_{10} has free radical activity and causes lung inflammation and epithelial injury. These data support our hypothesis concerning the mechanism for the adverse effects of particulate air pollution on patients with airway diseases. — *Environ Health Perspect* 105(Suppl 5):1279–1283 (1997)

Key words: air pollution, reactive oxygen species, oxidant, antioxidant, glutathione, epithelial cells

Introduction

Particulate matter with an aerodiameter of ≤10 μm (PM_{10}) is a ubiquitous pollutant of urban air (*1*). Numerous epidemiologic studies have shown that particulate air pollution is associated with increased morbidity and mortality (*2*). PM_{10} levels in ambient air are associated with reductions in lung function (*3*) and hospital admissions for asthma (*4*) and for chronic obstructive pulmonary disease (*5*). In addition there is an association between PM_{10} levels and cardiovascular deaths (*2*). These associations have been demonstrated in diverse geographical locations (*1*) where the source of PM_{10} has varied from primarily industrial to mainly vehicle exhaust, which suggests that the exact composition of the particulate air pollution may not be critical.

Although epidemiologic evidence strongly supports an association between PM_{10} and adverse health effects, the mechanism is not understood (*6*). Moreover, the development of adverse effects with PM_{10} at such low airborne mass concentrations remains a puzzle. We hypothesized (Figure 1) that exposure to PM_{10} particles produces airway inflammation, increased airspace permeability, and interstitialization of the particles, thus enhancing the inflammatory response (*7*), which has been proposed for other inhaled particles as a result of their oxidant properties (*8*). This inflammation results in exacerbations of airway disease and also causes changes in the coagulation and rheology of blood cells through both local and systemic effects. This latter effect may be critical in precipitating cardiovascular events and hence deaths in a susceptible population (*7*). The purpose of this study was to test this hypothesis by measuring proinflammatory potential and the oxidant activity of PM_{10} in the lungs. We have also tested this hypothesis by comparing the effects of PM_{10} with those of fine (CB) and ultrafine carbon black particles (ufCB).

Materials and Methods

Particle Suspensions

PM_{10} particles were collected on glass fiber filters from a tapered element oscillating microbalance at the Edinburgh monitoring site of the U.K. Enhanced Urban Network. Such PM_{10} samplers have been established in several cities by the U.K. government. This sampler has an impactor that enables a collection of particles, of which at least 50% have a diameter of less than 50 μm. The filters were stored for up to 4 months until used as described below.

The PM_{10} filter was cut into small pieces and 0.8 ml phosphate-buffered saline (PBS) was added and vortexed for 20 sec. The filter was removed to avoid further contamination with filter fibers, and the suspension was then sonicated for 30 sec (Ultrasonic Cleaner BP-1, Burkard Scientific Sales Vineland, NJ). Since the extraction procedure produced a suspension of PM_{10} contaminated with small numbers of filter fibers (0–10 per light microscopic field at ×80 magnification), a filter fiber suspension (FFS) was prepared by sonicating an unused filter of the same type used to collect PM_{10} in PBS for 30 sec. This suspension contained greater than 300 fibers per light microscopic field (×80 magnification).

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Abbreviations used: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; BSA, bovine serum albumin; CB, fine carbon black particles; DMEM, Dulbecco's minimum Eagle's medium; FFS, filter fiber suspension; GSH, reduced glutathione; GSSG, oxidized glutathione; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PM_{10}, particulate matter with anerodynamic diameter of < 10 μm; NO, nitric oxide; TNF, tumor necrosis factor; ufCB, ultrafine carbon black particles.

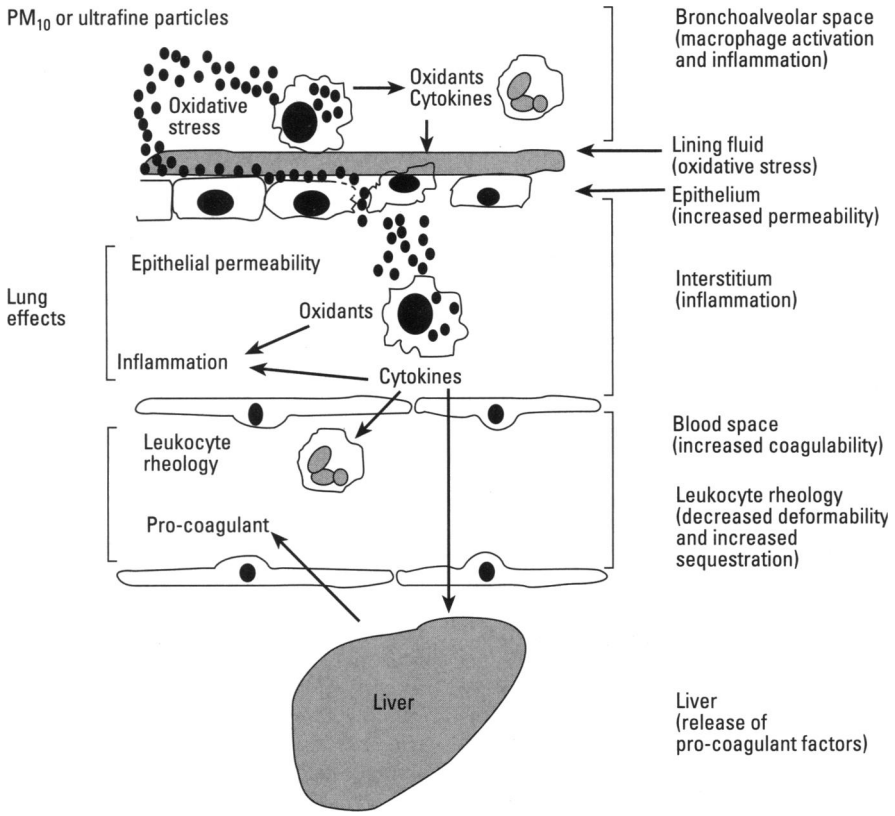

Figure 1. Diagramatic representation of a hypothesis to explain the mechanism for the harmful effects of particulate air pollution.

We calculated the mean weight of the particles on eight filters as 996 ± 182 μg (mean ± SEM). The method of preparation removed 20 to 50% by weight of the particles. Thus we estimate that between 199 to 498 μg of particles were present in 0.8 ml of PBS. Since 0.2 ml was instilled, this volume contained between 50 to 125 μg of particles. U.K. air quality standards have 50 $\mu g/m^3$ as an upper level of PM_{10}. The preparation was used within 24 hr.

The effects of instillation of PM_{10} in rat lungs were compared with instillation of those of CB (Degussa Huber NG90, diameter 200–250 nm) and ufCB (Degussa printex 19, diameter 20 nm; 125 μg in 0.2 ml PBS).

Intratracheal Instillation of Particle Suspensions

Syngeneic male Wistar-derived rats of the HAN strain, 12 weeks of age, were anesthetized with pentobarbitone, and 0.2 ml PBS–particle suspension was instilled intratracheally. The controls for these experiments were animals that did not receive any instillation and animals instilled with 0.2 ml PBS alone. Experiments were also carried out after intratracheal instillation of 0.2 ml filter fiber suspension.

Bronchoalveolar Lavage

Six hours after intratracheal instillation of particle suspensions, rats were sacrificed, and 4 ml PBS at 37°C was instilled and withdrawn from the lungs. After centrifugation this solution was referred to as bronchoalveolar lavage fluid (BALF). To obtain bronchoalveolar lavage (BAL) leukocytes, 4×8 ml PBS was used to wash the lungs and then collected in a universal tube. The cell suspension was spun and cell pellets were resuspended in Dulbecco's minimum essential medium (DMEM) medium (GIBCO, Paisley, UK) plus 0.2% low endotoxin–bovine serum albumin (BSA) (Sigma, Poole, UK) in which the cells from the first lavage were combined. The total number and differential count of BAL leukocytes were obtained. BAL leukocytes from control animals consisted of greater than 99% macrophages.

Soluble lung homogenate was prepared for measurement of reduced (GSH) and oxidized glutathione (GSSG). Postlavage lungs were resected and blotted dry. One gram of lung tissue was randomly sampled from all lung lobes. The samples were homogenized in 5% sulfosalicylic acid and the supernatant was then diluted in 0.1 M potassium phosphate buffer.

Collection of Cell Culture Supernatant

BAL leukocytes from control rats and rats after intratracheal instillation of PM_{10} were cultured in DMEM + 0.2% BSA at a concentration of 1×10^6 per ml for 24 hr in DMEM. Thereafter the supernatant was collected for the measurement of nitrite and tumor necrosis factor (TNF) as described below.

The A549 human type II alveolar epithelial cell line was purchased from ECACC (Salisbury, UK) and maintained in DMEM containing 10% fetal calf serum. To assay particle-induced A549 epithelial cell permeability and changes in glutathione, we co-incubated the cells with particle suspensions in DMEM + 2% BSA for 6 hr.

Measurement of Epithelial Permeability *in Vivo* and *in Vitro*

Rat lung epithelial permeability was assessed as the total protein concentration in BALF (*9*). This technique produced results similar to measurements of airspace epithelial permeability assessed as the passage of ^{125}iodine-labeled BSA from airspace to blood (*10*). Protein concentrations were determined by incubating BALF with Biorad solution (BioRad, Munich, Germany) for 10 min at room temperature. The absorbence was read at 595 nm on a Unicam 8700 series spectrophotometer (Unicam, Cambridge, UK). Protein concentration was determined by comparison with a standard curve for BSA.

As a model of airspace epithelium, the permeability of A549 type II epithelial cell monolayers was determined using a modification of a technique that we developed previously (*10*). However, instead of ^{125}I-BSA, we used unlabeled BSA in the assay. Briefly, A549 cells were cultured on Nunc tissue culture inserts (GIBCO) in a 24-well plate to form cell monolayers. The monolayers were incubated with particle suspensions for 6 hr. The media in both inserts and wells were replaced with PBS followed by the addition of 1 mg of BSA into the insert. Thirty minutes later, PBS in the wells was sampled and albumin concentrations were determined.

Tumor Necrosis Factor, Lactate Dehydrogenase, and Nitrite Assays

Tumor necrosis factor activity in BALF and supernatant from cell monolayers were measured using the L929 cell bioassay as described previously (*9*). Lactate dehydrogenase (LDH) concentrations were assessed using the method of Bergmeyer and co-workers (*11*).

Nitric oxide (NO) generation was determined as accumulated nitrite measured by a modified microplate assay using the Griess reagent (*12*).

Measurement of GSH and GSSG

The total cellular GSH concentration was assayed by the GSSG-reductase–DTNB recycling procedure as described previously (*10*). To measure GSSG, GSH in the samples was first depleted by incubation with 2-vinylpyridine followed by the GSSG-reductase–DTNB recycling procedure. GSH concentrations were then calculated by subtracting GSSG values from total GSH values. GSH and GSSG values were determined by comparison with GSH and GSSG (Sigma) standard curves.

Statistical Analysis

Results were expressed as mean ± SEM. Differences between mean values were assessed by analysis of variance.

Results

Intratracheal instillation of PM_{10} caused neutrophil influx in rat lungs 6 hr after instillation, which accounted for 10 to 15% of the total BAL leukocyte numbers (Figure 2). Compared with animals that had instillations of PBS, CB instillations produced a small but significant neutrophpil influx. However, the greatest inflammatory cell influx occurred after instillation of ufCB (Figure 2). In this case, neutrophils accounted for 40% of the total BAL leukocyte count. BAL leukocytes obtained 6 hr after PM_{10} instillation produced greater amounts of TNF and NO in culture compared with BAL leukocytes from PBS-instilled control animals (Figure 3). Although inflammatory BAL leukocytes showed a greater potential to produce TNF and NO in culture, TNF and NO in BALF were not significantly different 6 hr after PM_{10} instillation compared with BALF levels in PBS-instilled control animals (Table 1).

Table 1. TNF, NO, and LDH levels in rat lung BALF 6 hr after intratracheal instillation of PM_{10}.

	TNF, U/ml	Nitrite, μM/ml	LDH, U/ml
PBS control	0	12.9±3.9	13.0± 2.0
PM_{10}	8.0±5.8	10.5±1.0	453.0±52.3***

Mean ± SEM of three rats. ***$p<0.001$ compared with PBS control values.

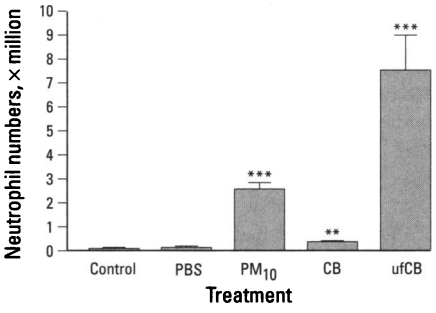

Figure 2. The number of neutrophils in BAL from rats 6 hr after intratracheal instillation of PM_{10}, CB , and ufCB. The results in rats that had no instillation (control) or instillation with PBS are shown for comparison. Histograms and bars represent the mean and SEM of three to six animals. **$p<0.01$, ***$p<0.001$ compared with PBS.

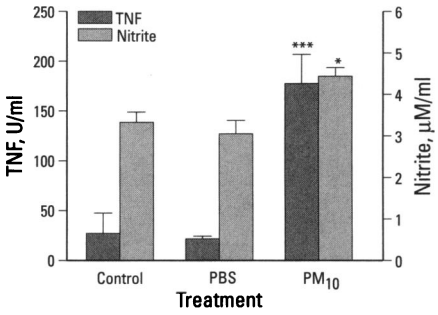

Figure 3. The effect of intratracheal instillation of PM_{10} on TNF and NO production by BAL leukocytes in culture. The results in rats that had no instillation (control) or instillation with PBS are shown for comparison. Histograms and bars represent the mean and SEM of three experiments. *$p<0.05$, ***$p<0.001$ compared with PBS.

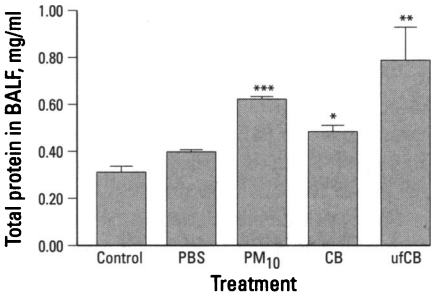

Figure 4. The effect of intratracheal instillation of PM_{10}, CB, and ufCB carbon black on rat lung epithelial permeability *in vivo*, measured as total protein values in BALF 6 hr after instillation. The results in rats that had no instillation (control) or instillation with PBS are shown for comparison. Histograms and bars represent the mean and SEM of three to six animals. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared with PBS.

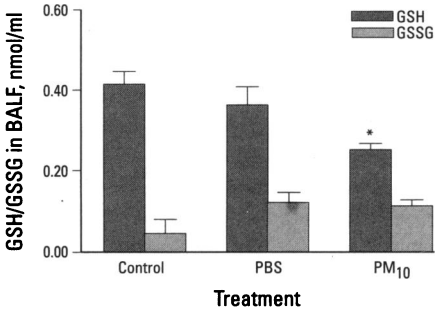

Figure 5. The effect of intratracheal instillation of PM_{10} on GSH and GSSG concentrations in BALF 6 hr after instillation in rat lungs. Histograms and bars represent the mean and SEM of three animals. *$p<0.05$ compared with PBS.

PM_{10} increased airspace epithelial permeability 6 hr after instillation, as shown by elevated total protein levels in BALF compared with PBS-instilled control animals (Figure 4). At this time point, LDH levels were higher than those in control BALF (Table 1). As with the influx of inflammatory leukocytes, the greatest increase in airspace epithelial permeability occurred after instillation of ufCB. CB produced a lesser increase in epithelial permeability than PM_{10} or ufCB (Figure 4).

Addition of PM_{10} to A549 type II alveolar epithelial monolayers *in vitro* increased their permeability to BSA (penetrated BSA control 0.10 ± 0.02; PM_{10} 0.20 ± 0.02 mg/ml; $p<0.01$). This increased epithelial permeability was not due to cell death, as monolayers of A549 cells incubated with PM_{10} for 6 hr did not release increased amounts of LDH (control LDH 23.5 ± 2.5; PM_{10} LDH 21.0 ± 7.1 U/2 million cells; $p>0.05$).

Intratracheal instillation of PM_{10} decreased GSH without any significant change in GSSG in BALF 6 hr after instillation, compared with PBS-instilled animals (Figure 5). However, GSH and GSSG levels in lung tissue were the same in PBS- and PM_{10}-treated rats (data not shown).

To clarify the role of fiber contamination in the activity of the PM_{10} suspension, we compared the effects of PM_{10}, FFS, and PBS instillations in the rat lung. Table 2 shows that FFS did not significantly alter

Table 2. The effect of intratracheal instillation of PBS or FFS on BAL leukocyte components, epithelial permeability, and GSH/GSSG in BALF 6 hr after intratracheal instillation in rat lungs.

Treatment	Neutrophil number, $\times 10^6$	Protein in BALF, mg/ml	GSH in BALF BALF, nmol/ml
PBS	0.68 ± 0.28	0.57 ± 0.03	0.44 ± 0.08
FFS	0.58 ± 0.15	0.55 ± 0.11	0.96 ± 0.08[a]

Mean ± SEM of three rats for each group. [a]$p < 0.01$ compared with PBS control.

BAL leukocyte components compared with PBS control 6 hr after intratracheal instillation, nor did it increase epithelial permeability, measured as total protein in BALF. Instillation of fiber suspension increased GSH levels in BALF but did not increase the permeability of A549 cell monolayers to BSA *in vitro* (penetrated BSA: control, 0.06 ± 0.01 mg/ml versus fiber suspension 0.05 ± 0.01) mg/ml, $p > 0.05$).

Discussion

These studies show that 6 hr after intratracheal instillation PM_{10} produced an acute neutrophil influx into the airspaces, with accompanying increased epithelial permeability. This inflammation and increase in epithelial permeability could have been due in part to a direct PM_{10}-induced epithelial injury, which is reflected in the elevated LDH concentrations in BALF. The ability of PM_{10} to cause epithelial permeability was confirmed *in vitro* by the increased transfer of albumin across epithelial monolayers in the presence of PM_{10}.

We found that the PM_{10} suspension was contaminated with a small number of fibers derived from the filter during the preparation procedure. We therefore assessed the ability of FFS alone, which contained at least 30 times more fibers than the PM_{10} suspension, to cause proinflammatory effects when instilled into rat lungs. These studies showed that fibers alone do not cause a neutrophil influx into the airspaces, or increased epithelial permeability, or decreased GSH levels in BALF. Thus we conclude that PM_{10} is responsible for the observed inflammatory effects in the lungs and not the presence of a small amount of filter fiber contamination.

Many particles, such as quartz (*13*), coal mine dust (*14*), asbestos (*15*), and ultrafine titanium dioxide (TiO_2) (*8*), that cause pathological effects in the lungs have detectable, but variable, amounts of free radical activity at their surfaces. Furthermore, the role of iron in producing the highly injurious hydroxyl radical has been suggested as a unifying theme in particle toxicity (*16*). Support for the role of iron in the oxidant effects of PM_{10} comes from preliminary studies from our laboratory indicating that the free radical activity of PM_{10} is abolished in the presence of the iron chelator desferrioxamine (*17*).

This effect may reflect the surface chemistry of the ultrafine component (*18*). We noted that the supernatant from high-speed centrifugation of PM_{10} suspension to clarity, which presumably contained only the ultrafine components, still caused the same degree of plasmid DNA scission (*17*). This strongly suggests that ultrafine particles provide the bulk of free radical activity of PM_{10}.

Our hypothesis that ultrafine particles have free radical activity is derived from our previous studies showing the oxidant potential of another ultrafine particle, TiO_2. This material has free radical activity in the ultrafine form (20 nm in diameter) but is inert as larger sized particles (250 nm) (*8*).

Further evidence in support of the contention that it is the free radical activity of PM_{10} that is responsible for its biological activity *in vivo* is shown by changes in the important lung antioxidant glutathione. PM_{10} decreased GSH but had no effect on GSSG levels in BALF after instillation. Furthermore, intracellular GSSG/GSH ratios were not affected by PM_{10} either in lung tissue *in vivo* or in epithelial cells *in vitro*, at least at the single time point when measurements were made. It is possible that such changes may have occurred at earlier time points, resulting in compensatory mechanisms, such as upregulation of the genes involved in GSH synthesis, after exposure to cigarette smoke, as we have reported (*19*).

Analysis of the exact composition of the PM_{10} sample that we used is not yet available. However, the composition of PM_{10} obtained from other sources indicates that carbonaceous material makes up 50% of the mass of PM_{10} (*20*). Therefore, we compared the effects of instillation of both CB and ufCB with those of PM_{10}, in similar doses, in the rat lung. These studies show that ufCB produce similar qualitative but greater quantitative proinflammatory effects to those of PM_{10} in the rat lung. The greater inflammatory effect of ufCB could have been anticipated from our hypothesis, as ufCB is composed entirely of ultrafine particles; this is not the case for PM_{10}, which has only 50% of its particles less than 10 μm in aerodiameter. This supports our contention that the ultrafine component of PM_{10} has the greatest inflammatory potential.

A decrease in lung GSH is associated with increased epithelial permeability caused by cigarette smoke, which has enormous oxidant potential (*10*). The present study showed a decrease in GSH in BALF but not in lung GSH after PM_{10} instillation. Further studies on the time course of changes in lung GSH are required to determine if a similar mechanism applies to the effects of PM_{10}. Other candidate inflammatory mediators may be involved in increasing epithelial permeability, such as TNF (*9*) and NO (*21*). Data from the present study do not show increased NO or TNF in BALF in association with a neutrophil influx into the airspaces after instillation of PM_{10}. However, *in vitro* BAL leukocytes from PM_{10}-treated rats produced significantly more NO and TNF in culture than those from control BAL cells. We believe that the release of these mediators results from the effect of loading the cells with particles (*22*) but also from PM_{10}-induced oxidant stress (*23*). In addition, there is an interaction between these two inflammatory mediators in that TNF can stimulate NO production (*24*). The absence of detectable TNF and NO levels in BALF from PM_{10}-treated rats compared with control animals is likely a result of the presence of inhibitors in BALF.

These studies are preliminary and are limited because of the lack of the availability of large quantities of PM_{10}. As a result, we were only able to study an animal model of instillation rather than the preferred inhalation model, which would be more relevant to environmental exposures. Thus, comparative calculations of the doses relative to environmental exposures are difficult. Therefore, in this preliminary study, where only one dose and one time point could be investigated because of the availability of PM_{10}, we opted to study a dose that was higher than environmentally plausible.

However, this study does provide evidence that PM_{10} has oxidant activity and causes an inflammatory response and epithelial injury in the lungs. These data provide support for our hypothesis (*7*) of the role of PM_{10} in exacerbating airway diseases.

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