

AIRWAY HYPERRESPONSIVENESS CAUSED BY AEROSOL EXPOSURE TO RESIDUAL OIL FLY ASH LEACHATE IN MICE

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Particulate air pollution is associated with exacerbation of asthma and other respiratory disorders. This study sought to further characterize the pulmonary effects of residual oil fly ash (ROFA), an experimentally useful surrogate for combustion-derived particulates in ambient air. Mice were exposed to aerosols of the soluble leachate of residual oil fly ash (ROFA-s). Physiologic testing of airway function (non invasive plethysmography) showed increased Penh, an index of airway hyperresponsiveness (AHR), in a time- and dose-dependent manner after exposure to ROFA-s. BAL analysis showed a minor influx of neutrophils, which was maximal at 12 h after exposure and essentially resolved by the time point of maximal AHR (48 h after exposure). The AHR caused by ROFA-s was reproduced by a mixture of its major metal components (Ni, V, Zn, Co, Mn, Cu) but not by any individual metal alone. Systemic pretreatment of mice with the antioxidant dimethylthiourea abrogated ROFA-s-mediated AHR. Analysis of mice of varying ages showed that ROFA-s had no marked effect on airway responsiveness of 2-wk-old mice, in contrast to the AHR seen in 3- and 8-wk old mice. ROFA-s-mediated AHR was unchanged in neurokinin 1 receptor knockout mice and in mice treated with a neurokinin antagonist, arguing against a role for this mediator in ROFA-s-mediated effects. Data indicate that ROFA-s mediates AHR in mice through antioxidant-sensitive mechanisms that require multiple metal constituents. Maturational differences in susceptibility to ROFA-induced AHR may be useful for further studies of mechanisms of particle effects.

Elevated levels of air pollution are linked epidemiologically to increased morbidity from asthma, pneumonia, and other respiratory-tract disorders (Becker & Soukup, 1998, 1999, Braun-Fahrlander et al., 1992; Gong, 1992; Nicolai, 1999). In some cases, air pollution components may cause respiratory symptoms even in normal subjects (Becker & Soukup, 1998). For example, ozone causes airway hyperresponsiveness (AHR) in both human subjects and animal models, not only in asthmatics but also in normal individuals (Bhalla, 1999; Shore et al., 2001). Recent epidemiologic studies identified an important association between levels of particulate matter of respirable size (PM_{2.5}) and asthma morbidity (Goldsmith & Kobzik, 1999). Experimental approaches to potential mechanisms for particle effects include use of well-defined surrogate particles, such as residual oil fly ash

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(ROFA). Dye et al. (1997) reported that ROFA causes epithelial injury of airways and lungs. The soluble metal fractions of the ROFA play an important role in the mechanism of this phenomenon (Broeckaert et al., 1997, 1999; Dreher et al., 1997). ROFA *in vitro* enhances inflammatory responses (Goldsmith et al., 1998; Stringer & Kobzik, 1998). In addition, ROFA can potentiate inflammatory processes *in vivo* in animal models. Prior work from this laboratory demonstrated that exposure to aerosolized ROFA leachate increases airway hyperresponsiveness in a murine asthma models of very young mice (Hamada et al., 1999). In these experiments, a control group exposed to only ROFA leachate (without allergen challenge) did not show AHR upon methacholine challenge. In contrast, Gavett et al. (1997) reported that ROFA itself could cause AHR in normal rats. The present study sought to investigate the effect of aerosolized exposure to ROFA leachate on airway physiology and pulmonary inflammation in normal mice of varying ages.

MATERIALS AND METHODS

Animals

Seven- to 8-wk-old female BALB/c mice weighing approximately 20 g were obtained commercially from Harlan Sprague Dawley (Indianapolis, IN) and fed a commercial pelleted mouse feed and given water *ad libitum*. The mice were housed in an animal facility that was maintained at 22–24°C with a 12-h dark/light cycle. Two- and 3-wk-old mice weighing approximately 8–12 g were also purchased with their mother mice and housed as described (Hamada et al., 1999). Neurokinin receptor 1 knockout mice (8–10 wk-old, weighing 25 g, BALB/c background) were generously provided by Dr. Norma Gerard (Lu et al., 1997).

Aerosol Exposure to Residual Oil Fly Ash Leachate

A single sample (1 kg) of residual oil fly ash (ROFA), obtained from the precipitator unit of a local power plant, was generously provided by Dr. John Godleski (Harvard School of Public Health, Boston). ROFA was suspended (100 mg/ml in phosphate-buffered saline (PBS), pH 7.4) and sonicated for 10 min. After sitting for 30 min at room temperature, the ROFA suspension was incubated at 37°C with rotation for 4 h, and then centrifuged at 3000 × *g* for 10 min. The supernatant (leachate) was removed and diluted (10, 50, or 100 mg/ml in PBS) for aerosol exposure. Mice were exposed to a nebulized aerosol of ROFA-s leachate (ROFA-s) for 30 min within individual compartments of a mouse “pie” chamber (Baintree Scientific, Baintree, MA) using a Pari IS2 nebulizer (Sun Medical Supply, Kansas City, KS) connected to air compressor (PulmoAID, DeVilbiss, Somerset, PA) (Rudmann et al., 2000). Control mice were exposed to PBS alone.

To investigate the time course of both physiological and pathologic responses, analyses were performed at 6, 12, 24, 48, 72, and 120 h after exposure to 50 mg/ml of ROFA-s. To examine the dose-response profile for

ROFA-s, effects of 10, 50, and 100 mg/ml ROFA-s and PBS were compared using analysis at a time point showing maximal AHR, 48 h after exposure. Although ROFA was suspended and diluted in PBS (pH 7.4), the final ROFA-s (50 mg/ml) solution was acidic (pH 5.5).

To evaluate the effect of pH, mice were also exposed to neutralized (pH 7.2 adjusted with NaOH) ROFA-s and acidic (pH 5.5 adjusted with HCl) PBS. As described, ROFA-s was a supernatant of centrifuged suspension liquid after sonication and incubation; however, to exclude the possible effect of uncentrifuged or residual fine particles, filtered (0.2 μm) ROFA-s were also examined.

To study the effects of antioxidant dimethylthiourea (DMTU), mice were injected intraperitoneally with different doses of DMTU (5, 10, 50, or 100 mg/kg) 30 min before the exposure to ROFA-s (50 mg/ml). Some mice were treated with DMTU (50 mg/kg) at 12 or 24 h after exposure. DMTU and all other reagents, unless otherwise specified, were obtained from Sigma Chemical Co. (St. Louis, MO).

Elemental analysis was performed upon ROFA-s using inductively coupled plasma/mass spectrometry (ICP/MS) as previously described (Imrich et al., 2000). The effects of each individual major metal components of ROFA-s were evaluated. The same concentrations of nickel sulfate (NiSO_4 , Ni), vanadium sulfate (VSO_4 , V), zinc sulfate (ZnSO_4 , Zn), cupric sulfate (CuSO_4 , Cu), manganese sulfate (MnSO_4 , Mn), and cobalt chloride (CoCl_2) as in ROFA-s were dissolved in PBS and adjusted to pH 5.5. Mice were exposed to aerosols of each individual metal solution and the reconstituted mixture of all metal solutions for 30 min and then tested physiologically and pathologically at 48 h after the exposure.

To study the role of neurokinins in the physiological effects of aerosolized exposure to ROFA-s, neurokinin receptor knockout mice were exposed to ROFA-s (50 mg/ml) and evaluated as described next. In addition, normal BALB/c mice exposed to ROFA-s were treated with neurokinin 1 receptor antagonist (CP-099,994, 10 mg/kg) by intraperitoneal injection at 30 min before the exposure (Fahy et al., 1995; Nsa Allogho et al., 1997).

To evaluate maturation effects on AHR caused by ROFA-s exposure, 2- and 3-wk-old young mice were also exposed to ROFA-s (50 mg/ml) and evaluated, similarly to their adult counterparts already described.

Physiologic Analysis

Airway responsiveness of mice to increasing concentrations of aerosolized methacholine (Mch) was measured using whole-body plethysmography (Buxco, Sharon, CT), as previously reported (Goldsmith et al., 1999; Hamada et al., 1999). Briefly, each mouse was placed in a chamber and continuous measurements of box pressure/time wave were calculated via a connected transducer and associated computer data acquisition system. The main indicator of airflow obstruction, enhanced pause (Penh), which shows strong correlation with the airway resistance examined by standard evalua-

tion methods, was calculated from the box waveform (Hamelman et al., 1997). After measurement of baseline Penh, aerosolized PBS or Mch in increasing concentrations (3, 6, 12, 25, and 50 mg/ml) were nebulized through an inlet of the chamber for 1 min, and readings (Penh measurements) were taken for 9 min after each dose. Penh values for the first 5 min after each nebulization were averaged and used to compare results across treatment groups and individual mice.

Pathologic Analysis

After physiologic testing, mice were euthanized with sodium pentobarbital (Veterinary Laboratories, Lenexa, KS). The chest wall was opened and the animals were exsanguinated by cardiac puncture. Serum was prepared and stored at -20°C . The trachea was cannulated, and bronchoalveolar lavage (BAL) was performed 5 times with 0.7 ml (empirically adjusted to 0.3 ml for 2-wk-old mice to achieve similar inflation of lungs) of sterile PBS instilled and harvested gently. Lavage fluid (recovery volume was about 80% of instilled) was collected, centrifuged at $300 \times g$ for 10 min, and the cell pellet was resuspended in 0.5 ml PBS. Total cell yield was quantified with a hemocytometer. BAL differential cell counts were performed on cytocentrifuge slides prepared by centrifugation of samples at 800 rpm for 5 min (Cytospin 2, Shandon, Pittsburgh, PA). These slides were fixed in 95% methanol and stained with Diff-Quik (VWR, Boston), a modified Wright-Giemsa stain, and in total 200 cells were counted for each sample by microscopy. Macrophages, lymphocytes, neutrophils, and eosinophils were enumerated. In a subset of experiments the lungs from 2-wk-old and adult mice were removed after euthanasia at 12 h after ROFA-s (50 mg/ml) or PBS exposure and stored at -70°C . The catalase activity within these tissue samples was assayed as described by Gonzalez-Flecha et al. (1993).

Statistical Analysis

Data are summarized as means \pm standard error. Differences among groups were evaluated using analysis of variance (ANOVA) with correction for multiple groups using Statview software (Abacus Concepts, Berkeley, CA). Statistical significance was accepted when $p < .05$.

RESULTS

Response to Aerosolized ROFA-s

Initial experiments measured the airway responsiveness of normal adult mice at different time points after an exposure to 50 mg/ml ROFA-s. This concentration was previously found to amplify airway hyperresponsiveness (AHR) in "asthmatic" mice (Hamada et al., 1999). Figure 1 shows the time course of increased Penh in response to methacholine challenge (AHR) in response to ROFA-s. For clarity, only the data for responses to methacholine at 6 and 12 mg/ml are shown; proportionally similar results were seen at higher methacholine concentrations (25, 50 mg/ml; data not shown). Mild

AHR was seen at 12 h after exposure. The maximum response was seen at 48 h after the exposure (Figure 1) to ROFA-s aerosols. Bronchoalveolar lavage (BAL) analysis revealed increased neutrophils within the lung and decreased macrophage recovery (Table 1). In contrast to the physiological response, neutrophil recruitment was maximal at an earlier time point (12 h).

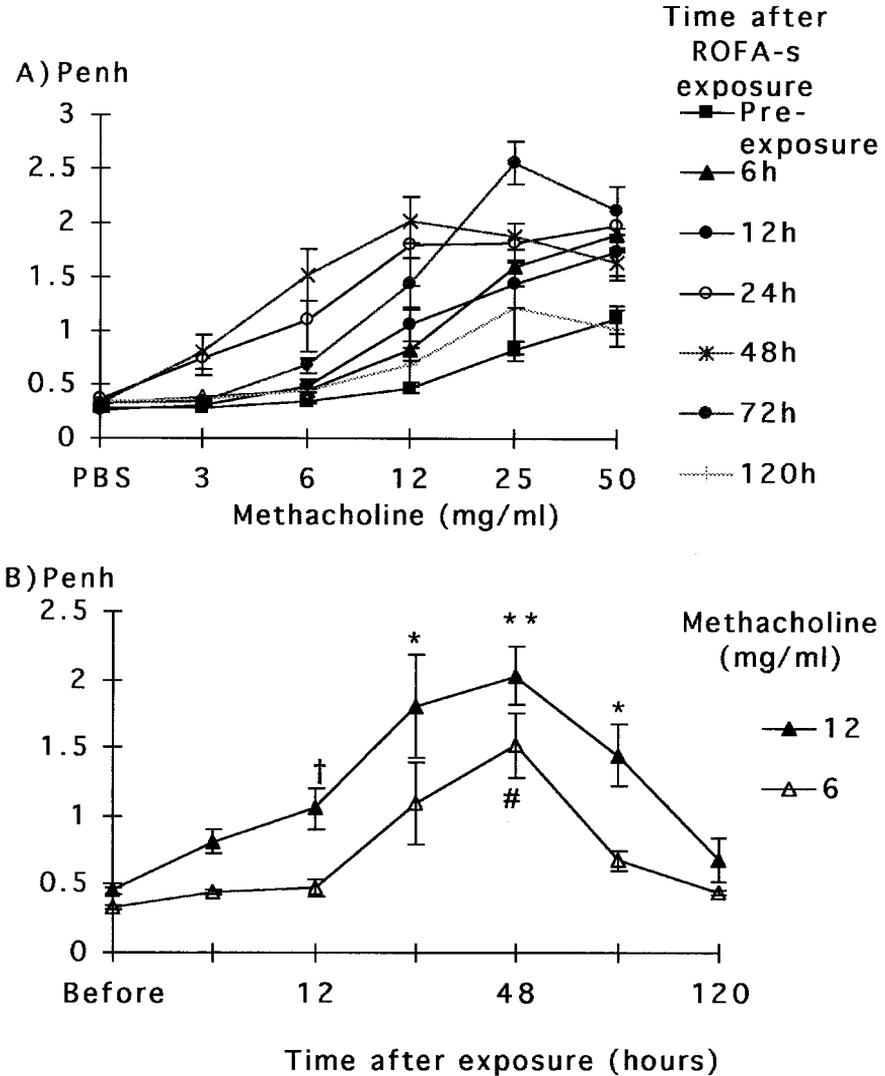


FIGURE 1. Results of physiological evaluation of airway function in mice exposed to ROFA-s aerosols. (A) Penh values obtained in response to increasing concentrations of methacholine. Cohorts of mice ($n \geq 6$ /group) were evaluated at varying time points after exposures. For clarity, (B) shows Penh values obtained after ROFA-s in response to two of the methacholine concentrations used. Asterisk indicates significant at $p < .05$ versus preexposure and 120-h time points; double asterisk, $p < .05$ versus preexposure and 12-, 72-, and 120-h time points; #, $p < .05$ versus all other time points; †, $p < .05$ versus preexposure; $n \geq 6$ /group.

TABLE 1. Summary of Cell Counts and Differentials Obtained After BAL Analysis of 8-wk-old Mice Exposed for 30 min to Aerosolized ROFA-s (50 mg/ml)

Cell type	ROFA-s, 6 h	ROFA-s, 12 h	ROFA-s, 24 h	ROFA-s, 48 h	PBS, 48 h
Total cells ($\times 10^5/\text{ml}$)	0.39 ± 0.02	0.44 ± 0.03	0.40 ± 0.03	0.42 ± 0.03	0.36 ± 0.02
Macrophages (%)	97.39 ± 0.39	91.94 ± 1.25^a	96.94 ± 0.53	97.28 ± 0.82	99.20 ± 0.34
Lymphocytes (%)	1.22 ± 0.19	0.89 ± 0.16	1.00 ± 0.27	0.64 ± 0.16	0.30 ± 0.12
Neutrophils (%)	1.39 ± 0.33	7.10 ± 1.23^a	1.97 ± 0.37	2.08 ± 0.84	0.50 ± 0.22
Eosinophils (%)	0.06 ± 0.04	0.08 ± 0.06	0.08 ± 0.05	ND	ND

Note. The data were obtained at the indicated time points after the aerosol exposures; $n \geq 18/\text{group}$. ND, not detected.

^aSignificant at $p < .05$ versus all others.

Results of a dose-response analysis using ROFA-s prepared from 10, 50, and 100 mg/ml ROFA) are shown in Figure 2. The two higher concentrations produced a similar increase in Penh in response to methacholine challenge, consistent with airway hyperresponsiveness (AHR). However, a lower concentration of ROFA-s (10 mg/ml) did not induce AHR in exposed mice (Figure 1). Unless otherwise noted, subsequent experiments analyzed effects of aerosol exposures to 50 mg/ml of ROFA-s at the 48-h time point.

ROFA Component Analysis

Table 2 shows the concentration within ROFA-s of a panel of potentially toxic metal components of ROFA. To test the effect, if any, of aerosol expo-

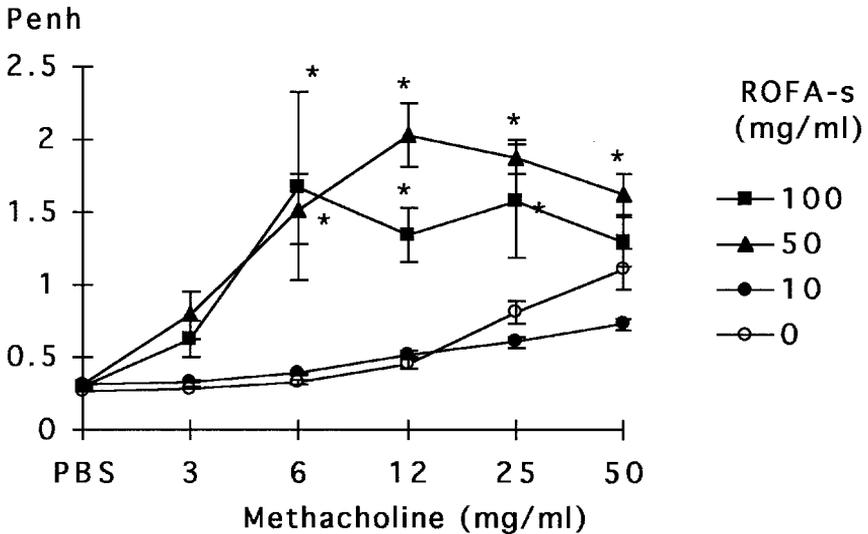


FIGURE 2. Results of physiological evaluation of airway function in mice exposed to aerosols of ROFA-s prepared from increasing concentration of ROFA (see methods). Asterisk indicates significant at $p < .05$ versus 0 and 10 mg/ml, $n \geq 4/\text{group}$.

TABLE 2. Metal Content Data Obtained by ICP/MS Analysis of ROFA-s (Prepared as 50 mg/ml Solution)

Metal	Metal content of ROFA-s ($\mu\text{g/ml}$)
Ni	341.2
V	323.4
Zn	232.3
Co	18.33
Mn	15.8
Cu	6.71
Cd	0.61
Fe	0.0

sure to these individual elements, solutions of the metals were prepared at concentrations identical to those found within ROFA-s (Table 2). Figure 3 summarizes effects on airway responsiveness in mice exposed to the each of six metal solutions. While no marked effects were seen with the individual elements, a reconstituted mixture of the panel of six reproduced the AHR seen with ROFA-s (Figure 3). After exposure to individual metals, only mice exposed to Ni showed a small increase of neutrophils ($2.8 \pm 1.0\%$). Mice exposed to the mixture of metals showed a low level of BAL neutrophils ($1.3 \pm 0.6\%$), similar to that seen after ROFA-s exposure (Table 1).

To determine the role, if any, of pH changes in the effect of ROFA-s on airway responsiveness, airway responses after exposure to neutralized (pH

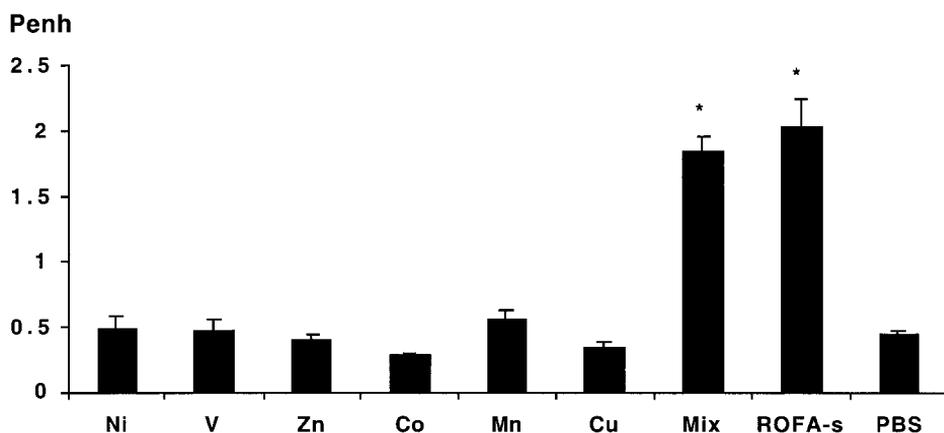


FIGURE 3. Results of physiological evaluation of airway function in mice exposed to aerosolized solutions of single metal components of ROFA, a mixture of all the single components, ROFA-s, or PBS. The solutions were prepared at the concentrations found in ROFA-s. Data presented was obtained in response to 25 mg/ml methacholine. Asterisk indicates significant at $p < .05$ versus all others, $n \geq 4$ /group.

7.2) ROFA-s and filtered ROFA-s were compared to those seen after standard ROFA-s (50 mg/ml, pH 5.5). Figure 4 shows that a similar degree of AHR was seen in animals exposed to either acidic or neutralized ROFA-s. Aerosol exposure to acidic PBS (pH adjusted; pH 5.5) exposure did not per se cause AHR (Figure 4).

Effects of Antioxidant Treatment

To investigate the role of oxidant stress in ROFA-s effects, the antioxidant dimethylthiourea (DMTU) was administered (ip) before exposure to ROFA-s (50 mg/ml). Figure 5 shows that DMTU pretreatment diminished AHR in a dose-dependent manner. For clarity, only the results of physiologic testing using methacholine at 6 and 12 mg/ml are shown. Similar results were seen with higher concentrations of methacholine (25 or 50 mg/ml, data not shown). The inhibition was statistically significant at 50 and 100 mg/kg DMTU. As noted earlier, neutrophil numbers in BAL fluid at 48 h after the exposure to ROFA-s are lower than those seen at 12 h after exposure. At these low levels, it is difficult to detect or interpret downward decreases. Nevertheless, after DMTU treatment, the level of neutrophils in BAL samples showed a trend consistent with the diminished AHR seen in these animals [percent neutrophils in BAL fluid: 1.8 ± 0.4 in PBS-treated group vs. 1.9 ± 0.5 , 0.8 ± 0.2 , 0.6 ± 0.2 , and 1.5 ± 0.2 in DMTU (5, 10, 50, and 100 mg/kg, respectively)-treated groups (mean \pm SE)].

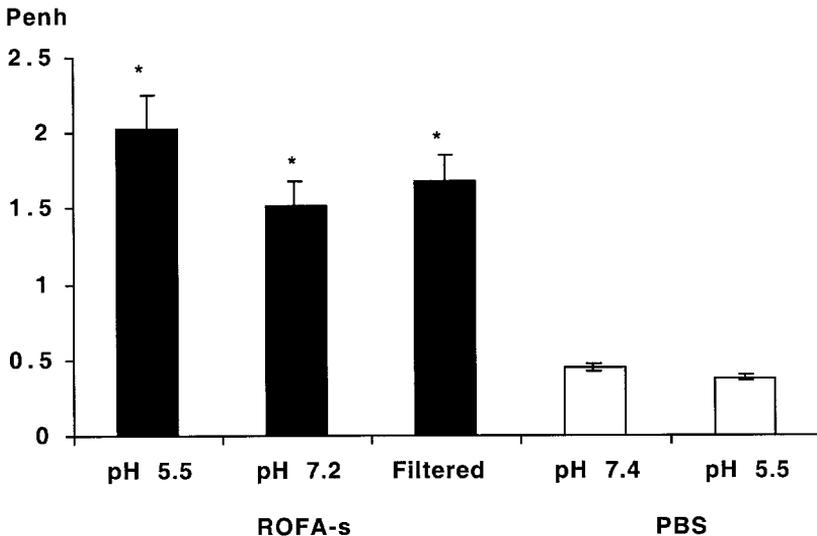


FIGURE 4. Evaluation of the effect of pH on the increased Penh seen with ROFA-s. Physiological evaluation of airway function was performed 48 h after exposure of mice to the aerosolized solutions listed on the x axis. Data presented were obtained in response to 50 mg/ml methacholine. Asterisk indicates significant at $p < .05$ versus PBS-exposed groups; $n \geq 6$ /group.

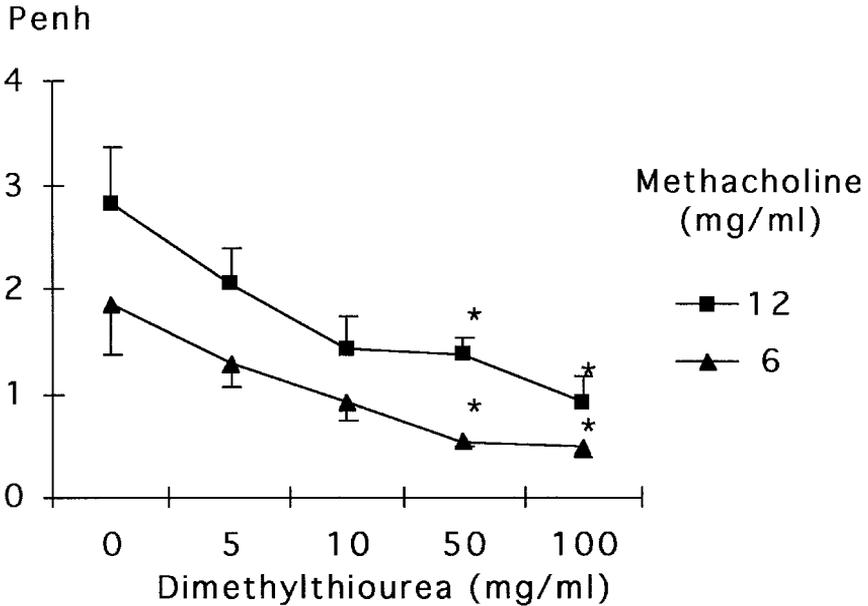


FIGURE 5. Evaluation of the effect of varying doses of ip dimethylthiourea on the increased Penh seen with ROFA-s. Physiological evaluation of airway function was performed 48 h after exposure of mice to ROFA-s (50 mg/ml). For clarity, data are presented for two of the methacholine concentrations used in these evaluations. Asterisk indicates significant at $p < .05$ versus PBS treatment, $n = 8/\text{group}$, except for 100 mg/ml where $n = 4/\text{group}$.

Development and Susceptibility to ROFA Effects

To investigate the effect of maturation on ROFA-s mediated AHR, the effect of ROFA-s aerosols (50 mg/ml) on younger mice (2 and 3 wk old) was compared to that seen in the 8-wk-old adult mice. Figure 6 shows that ROFA-s exposures that produce AHR in 8 week old mice have no marked effect on 2-wk-old mice. The ROFA-s aerosols begin to show an effect on AHR in 3-wk-old mice. No change in neutrophils was noted in the 2- or 3-wk-old mice, while the 8-wk-old mice did show a small number of neutrophils (see Table 1). Analysis of BAL cellularity in 2-wk-old mice at 6 and 12 h after ROFA-s revealed an initial inflammatory response at 6 h with rapid return to normal levels of neutrophils by 12 h (BAL PMNs % \pm SE, 6 and 12 h after ROFA-s respectively: 23 ± 0.6 ; $0.5 \pm .01$; $n = 5/\text{group}$).

The potential developmental differences between 2- and 8-wk-old mice that might affect responses to inhaled irritants include (1) differences in airway innervation (Haxhiu-Poskurica et al., 1991) and its regulation of airway caliber via neurogenic inflammation, and (2) differences in antioxidant enzyme levels that might modulate ROFA-mediated oxidant stress. To test the former possibility, mice with a genetic deletion in neurokinin receptor 1 were exposed to ROFA-s (50 mg/ml) prior to evaluation of airway respon-

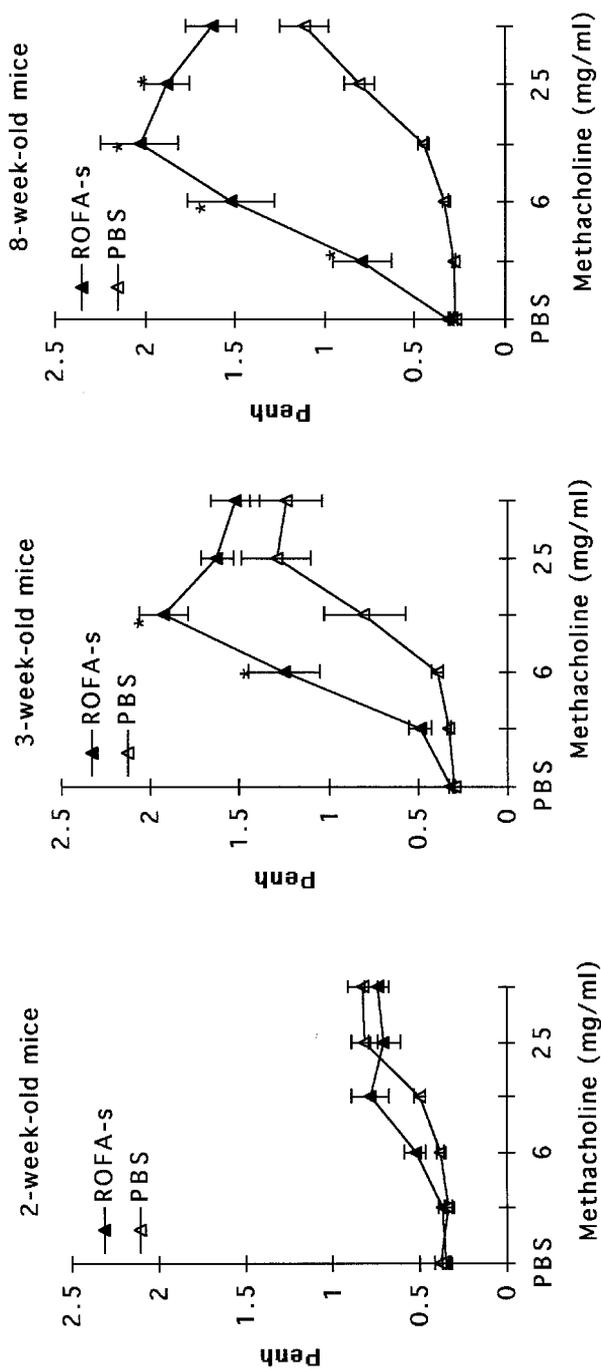


FIGURE 6. Comparison of the effect of ROFA-s on airway responsiveness (Penh) in mice of varying age (2, 3, and 8 wk old). Data presented was obtained 48 h after exposure to a 50-mg/ml solution. Asterisk indicates significant at $p < .05$ versus PBS, $n \geq 6$ /group.

siveness. The airway responsiveness of these mice was compared to wild-type (BALB/c) mice exposed to ROFA-s or PBS. Normal mice were also pre-treated with neurokinin 1 receptor antagonist (CP-099,994) prior to ROFA-s exposure. Figure 7 shows that ROFA-s produced a similar level of AHR in wild-type, NK1 receptor knockout mice, or mice treated with the NK1 receptor antagonist. To evaluate antioxidant status, catalase activity was measured 12 h after exposure to ROFA-s or PBS in lung tissues from young (2-wk-old) and adult (8-wk-old) mice (Figure 8). These assays showed similar levels of catalase activity after both treatments in the two age groups.

DISCUSSION

This study used aerosol exposures to a leachate of ROFA to investigate mechanisms of respiratory effects of particulate air pollution. The major findings include: (1) striking developmental differences in susceptibility to ROFA-s; (2) a discordance between the time course of pulmonary inflammation and AHR; and (3) a requirement for interaction between component metals to induce AHR. Abrogation of ROFA-s effects by systemic treatment with the antioxidant DMTU supports a role for oxidant-mediated pathways in the generation of AHR by ROFA.

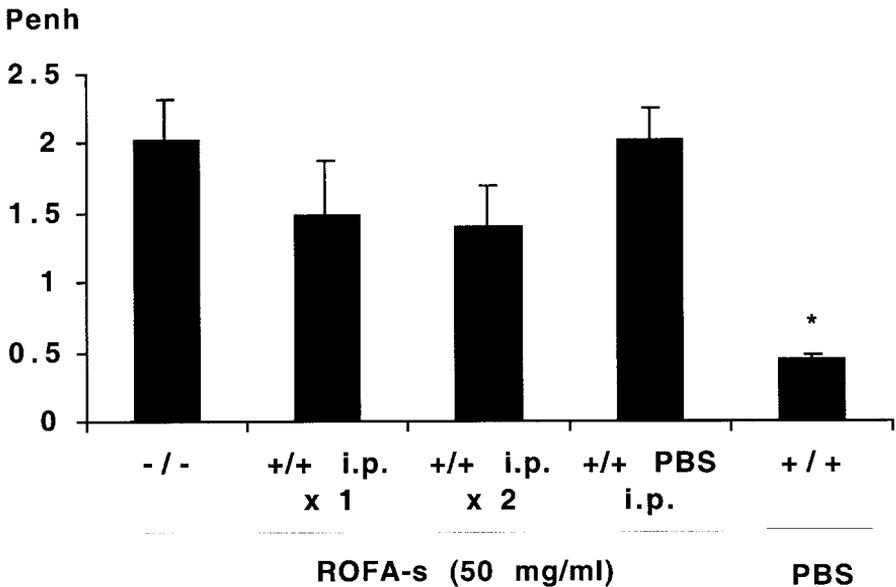


FIGURE 7. Evaluation of the effect of ROFA-s (50 mg/ml) on mice with a genetic deletion of neurokinin 1 receptor (-/-) or wild-type (+/+) mice treated ip with a neurokinin 1 antagonist. Data presented was obtained in response to 50 mg/ml methacholine. Asterisk indicates significant at $p < .05$ versus all other groups; $n \geq 4$ /group.

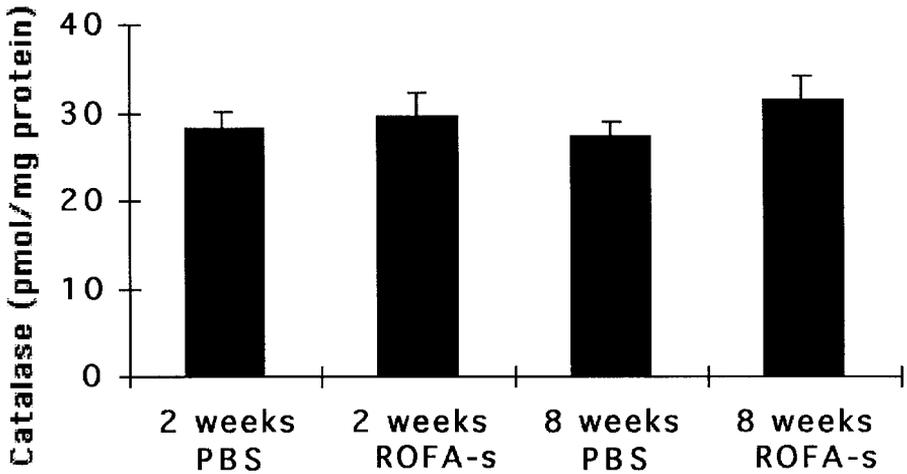


FIGURE 8. Measurement of catalase activity in 2- or 8-wk-old mice exposed to control PBS or ROFA-s aerosol shows no difference in this antioxidant among these groups; $n = 3/\text{group}$.

Particulate air pollution is associated with cardiopulmonary diseases including bronchial asthma (Dockery et al., 1993; Goldsmith & Kobzik, 1999). Previous studies have found that ROFA causes airway or pulmonary epithelial injury, and the mechanism(s) of this injury has been considered to be associated with the harmful effect of active oxygen species from transition metal components of ROFA (Broeckaert et al., 1999; Dreher et al., 1997; Dye et al., 1997). The soluble fraction of ROFA acts to increase AHR in a mouse model of asthma (Hamada et al., 1999). It has been reported to produce airway hyperresponsiveness in normal adult rats and lung inflammation in mice (Broeckaert et al., 1999; Gavett et al., 1997), but this laboratory observed an absence of AHR in very young mice exposed to ROFA leachate aerosols (Hamada et al., 1999). Hence this study sought to more rigorously characterize the effects of aerosol exposure in young and adult mice.

In adult mice, aerosolized exposure to ROFA leachate increased airway responsiveness as described in the text. The time courses of the physiological and pathological (inflammatory cell recruitment) changes were noteworthy. Although mild and transient recruitment of neutrophils was seen at 12 h after the exposure as the maximum in the lavage fluid, a physiological response was not prominent at this point. Rather, AHR became more pronounced and maximal in the 24–48 h after the exposure, after the decline of inflammatory cell numbers in the BAL analyses. Data indicate that oxygen radical species from aerosolized ROFA leachate play a pivotal role since pretreatment of antioxidant dimethylthiourea (DMTU) abrogated these responses. The antioxidant *N*-acetylcysteine also abrogated ROFA-mediated inflammatory responses in vitro (Stringer & Kobzik, 1998). Whether generation of these oxidants requires or is derived from the tran-

sient neutrophilia observed remains undetermined. We have previously observed robust neutrophil influx into the lungs (induced by lipopolysaccharide [LPS] aerosols) without development of AHR (Goldsmith et al., 1999).

There have been reports that soluble metal components of the ROFA leachate produce varying airway and lung injury (Broeckaert et al., 1999; Dreher et al., 1997; Kodavanti et al., 1998). The effects of vanadium (V), nickel (Ni), and iron (Fe) were considered to be important. The potential role of iron in oxidant injury is widely recognized. However, our ROFA leachate did not include iron (Table 1). Although aerosolized exposure to single soluble metal solutions (even acidic) failed to cause AHR, mixed metal solution could reproduce the same response produced by ROFA leachate. This indicates that interactive effects of metal constituents are required for the responses observed in this study. It is possible that these interactive effects derive from synergistic, antagonistic, or additive effects among the five metals studied.

Neurogenic inflammation in both human and experimental animal models plays an important role on airway hyperresponsiveness, including asthma (Barnes, 1996). A physiological response without detectable pathological changes, especially cellular inflammation, may reflect neurogenic inflammation. In these studies, aerosolized exposure to ROFA leachate induce relatively transient recruitment of neutrophils, with only a few inflammatory cells remaining at the time when the mice showed maximal AHR to methacholine challenge. Neurokinin receptor 1 knockout mice were used to investigate one pathway by which neurokinin, a key factor in neurogenic inflammation (Baluk, 1997; Barnes, 1996), might mediate the observed AHR. However, the neurokinin receptor 1 knockout mice revealed the same physiological response to ROFA-s as their wild-type counterparts. Moreover, treatment with a neurokinin antagonist (Fahy et al., 1995; Nsa Allogho et al., 1997) also did not decrease the AHR response. Hence the data do not support a mechanistic role for neurokinin receptor 1. The potential role of other receptor subtypes or other mediators, such as endotoxin (Ning et al., 2000), was not directly addressed in these experiments. However, ROFA-s had undetectable levels of endotoxin when tested by *Limulus* assay (data not shown), and LPS aerosols sufficient to cause neutrophilia did not cause AHR in other experiments (Goldsmith et al., 1999). Other limitations of this study include the differences in composition between ROFA and urban air particles and the more general concern that experimental studies (including this one) use toxicologic doses in their attempts to identify mechanistic pathways.

It was noteworthy that there was a different physiological and inflammatory response to aerosolized exposure to ROFA leachate seen in mice of varying age. In adult mice, ROFA leachate induced AHR. In contrast, AHR was not seen in 2-wk-old young mice, despite a robust and earlier neutrophilia. This laboratory has reported that 2-wk-old mice can show AHR after inhalation of aerosolized allergen (Hamada et al., 1999). Hence, the

absence of AHR to ROFA-s in the 2-wk-old mice in this study does not reflect a global inability to manifest or detect AHR. Since oxygen radical species are operative in the mechanisms of AHR by ROFA-s, the possibility that young mice might have relatively high activity of oxygen radical scavengers such as catalase in comparison with adult mice was investigated. However, catalase activity in the lung tissue of both young and adult was the same. Other potential antioxidants (e.g., superoxide dismutase, ascorbic acid, or glutathione) remain to be investigated. Hence, the mechanism(s) of the maturation-based differences in susceptibility to ROFA-s remain undetermined. The relative resistance of young mice to ROFA effects contrasts with other experimental and epidemiologic (Bhalla, 1999; Goldsmith & Kobzik, 1999; Shore et al., 2001) observations suggesting greater, not diminished, susceptibility of young subjects to inhaled pollutants. Future studies of the maturational transition from resistant to susceptible phenotype may provide insights into mechanisms of particle effects on the lung.

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