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In Vivo Evidence of Free Radical Formation in the Rat Lung after Exposure to an Emission Source Air Pollution Particle†

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Exposure to air pollution particles can be associated with increased human morbidity and mortality. The mechanism(s) of lung injury remains unknown. We tested the hypothesis that lung exposure to oil fly ash (an emission source air pollution particle) causes *in vivo* free radical production. Electron spin resonance (ESR) in conjunction with the spin trap α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (4-POBN) was used to detect radical adducts. Rats were instilled with 500 μg of either oil fly ash or saline. Twenty-four hours later, ESR spectroscopy of the chloroform extract from lungs of animals exposed to the oil fly ash gave a spectrum consistent with a carbon-centered radical adduct (hyperfine coupling constants $a^N = 15.0$ G and $a^H_\beta = 2.5$ G), while those spectra from lungs instilled with saline revealed a much weaker signal. This signal was reproduced by instilling animals with the soluble fraction of the oil fly ash, which contains soluble metal compounds. The same signal was observed after instillation of either a mixture of vanadium, nickel, and iron sulfates or $VOSO_4$ alone. We conclude that, after instillation of an air pollution particle in the rat, ESR analysis of lung tissue demonstrates *in vivo* free radical production. This generation of free radicals appears to be associated with soluble metals in the oil fly ash.

Introduction

Epidemiologic studies have established an association between exposure to air pollution particles and human mortality and morbidity. Episodes of acute particulate pollution in the Meuse Valley of Belgium in 1930, Donora, PA, in 1948, and London, England, in 1952 demonstrated that extremely high particulate levels can be associated with a dramatic increase in mortality. Recent epidemiologic studies suggest that current levels of particles in American cities similarly increase mortality (*1*). The evidence of an association between particles and mortality has been documented in numerous investigations of at least 16 cities and is remarkably consistent. Much of this excess mortality is attributed to a respiratory injury (*2*, *3*). In addition to increases in mortality, exposure to these particles has been associated with morbidity including increased respiratory symptoms (*4*, *5*). Elevated levels of air pollution can affect airway reactivity with decrements in several indices of pulmonary function (*6*, *7*) especially in those patients with preexisting asthma. The National Health and Nutrition Examination surveys demonstrated a relationship between ambient levels of total suspended particles and both forced vital capacity and forced expiratory volume in 1 s across a wide geographic and sociologic spectrum (*7*). Air pollution particles are also associated with increased incidences of both pulmonary infections and hospitalization for respiratory disease (*4*, *8*, *9*). These health effects are observed at particle concentrations below the current air quality standard of 150 μg/m^3 set to protect public health.

The mechanism(s) of lung injury after exposure to air pollution particles is not known. Injury has been postulated to be mediated by ultrafine particles (*10*), biological agents (e.g., endotoxin) (*11*), acid aerosols (*12*), and polyaromatic hydrocarbons (*13*). Oxidant generation catalyzed by metals associated with air pollution particles could also account for lung injury following exposure to air pollution particles. The *in vitro* generation of oxygen-derived free radicals by both emission source and ambient air pollution particles has been documented (*14*, *15*). The *in vivo* formation of free radicals in tissues after exposure to air pollution particles has not been demonstrated. We tested the hypothesis that exposure to an emission source air pollution particle is associated with *in vivo* free radical production.

Materials and Methods

Materials. The oil fly ash used had a mass median aerodynamic diameter of 1.95 ± 0.18 μm and was acquired from Southern Research Institute (Birmingham, AL). It was collected in Florida using a Teflon-coated fiberglass filter downstream from the cyclone of a power plant which was burning a low sulfur number 6 residual oil (collection temperature of 204 °C). The animals employed in all studies were 60-day-old, male Sprague−Dawley rats (Charles River Breeding Laboratories, Raleigh, NC). Vanadium(IV) sulfate oxide ($VOSO_4 \cdot 4H_2O$) was acquired from Alfa (Ward Hill, MA). All other materials were

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obtained from Sigma unless otherwise specified.

Organic and Mineral Ash Analyses. An elemental analysis of carbon and hydrogen in the oil fly ash was accomplished employing infrared and thermal conductivity assays (Galbraith Labs, Knoxville, TN) (*16*). Nitrogen content of the air pollution particle was measured using thermal conductivity after acid digest (Galbraith Labs, Knoxville, TN) (*16*). Mineral ash analysis was performed using ICPES (Perkin Elmer 40, Norwalk, CT) after acid digest (*16*).

The oil fly ash was agitated in distilled water for 1 h and centrifuged at 1200*g* for 10 min, and the supernatant was removed. The insoluble fraction of the air pollution particle was also analyzed for organic content and mineral ash (*16*). The soluble fraction is assumed to be the difference between the oil fly ash and its insoluble component.

***In Vivo* Inflammatory Injury.** Lung injury following intratracheal instillation of oil fly ash was investigated using 60-day-old (250−300 g), male Sprague−Dawley rats (n = 6/exposure). After anesthesia with halothane (Aldrich, Milwaukee, WI), either dust (1.0 mg total dose) in 0.5 mL of saline or 0.5 mL of normal saline (0.9% sodium chloride) was instilled. In addition, 20.0 mg of dust was agitated in 10.0 mL of normal saline and centrifuged at 1200*g* for 10 min, and the supernatant was removed. The insoluble fraction was resuspended in 10.0 mL of normal saline. Either the supernatant (0.5 mL) or the suspension of insoluble fraction (0.5 mL) was also instilled in rats. The pH values of these suspensions were 3.4 (oil fly ash), 6.8 (insoluble fraction), and 3.3 (soluble fraction), while the pH value of the normal saline was 6.9. Twenty-four hours after exposure, rats were anesthetized with halothane, euthanized by exsanguination, and tracheally lavaged. The volume of saline injected was determined from published allometric equations and equaled ∼90% of the total lung capacity (35 mL/kg of body weight). Saline was withdrawn after a 3-s pause, reinjected an additional two times with similar delays, and then stored on ice. Employing a modified Wright's stain (Diff-Quick stain, American Scientific Products, McGaw Park, IL), neutrophils were enumerated by counting 200 cells/animal at a magnification of 400×, and values were expressed as the percentage of total cells recovered. Lavage protein was determined using the Pierce Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) and modified for automated measurement (Cobas Fara II centrifugal analyzer, Roche Diagnostic, Montclair, NJ). Bovine serum albumin served as the standard.

***In Vivo* ESR Studies.** Animals were instilled with either 500 μg of oil fly ash in 0.5 mL of saline (n = 7), the equivalent mass of the insoluble component in 0.5 mL of saline (n = 3), the equivalent mass of the soluble fraction in 0.5 mL of saline (n = 3), or 0.5 mL of saline alone (n = 7). Twenty-four hours after exposure, rats were intraperitoneally injected with an aqueous solution of 8 mmol/kg of body weight α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (4-POBN).[1] After 1 h, the animals were anesthetized with 25 mg/kg of body weight pentobarbital (Abbott Laboratories, North Chicago, IL) intraperitoneally and euthanized by exsanguination. The entire lungs were excised and homogenized in 1.0 mL of 50 mM 2,2′-dipyridyl, 3.0 mL of 2:1 chloroform:methanol, 1.0 mL of chloroform, and 1.0 mL of deionized water. The homogenizer (Janke Kunkel, Germany) was used at maximum power with the tissue surrounded by an ice bath. 2,2′-Dipyridyl was included to inhibit *ex vivo* reactions initiated by iron. After centrifugation of the homogenate at 3000 rpm for 10 min (Beckman TJ-6), the chloroform layer was isolated. ESR spectra were recorded immediately at room temperature using a quartz flat cell and a Varian E-109 ESR spectrometer equipped with a TM_{110} cavity and operating at 9.33 GHz, 20-mW microwave power, and 100-kHz modulation frequency. Spectra were recorded on an IBM-compatible computer interfaced with the spectrometer. The ESR spectral simulations were performed using an automatic optimization procedure.

Animals (n = 3) were also instilled with a "synthetic oil fly ash", which is a mixture of vanadyl, nickel, and iron(III) sulfates in concentrations approximately equivalent to those included in 500 μg of oil fly ash (0.5 mL of the synthetic oil fly ash, which included 3700 μM $VOSO_4$, 640 μM $NiSO_4$, and 630 μM $Fe_2(SO_4)_3$). This synthetic oil fly ash was made up in normal saline and had a pH value of 4.32. Twenty-four hours later, 4-POBN was provided, the lungs were excised and homogenized, and the ESR spectra were recorded. Finally, this was repeated after the animals were instilled with 0.5 mL of either 1.0 mM $VOSO_4$ (n = 3), 1.0 mM $NiSO_4$ (n = 3), or 1.0 mM $Fe_2(SO_4)_3$ (n = 3) in normal saline (pH values of 3.82, 4.47, and 2.75, respectively).

[1] Abbreviations: electron spin resonance, ESR; α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone, 4-POBN.

Table 1. Organic and Mineral Ash Analysis (%; w/w) of the Oil Fly Ash

	oil fly ash	insoluble fraction
Organic Analysis		
carbon	1.17	7.44
hydrogen	1.39	<0.5
nitrogen	<0.5	<0.5
sulfur (pyritic)	0.09	<0.01
Mineral Analysis		
phosphorus pentoxide	0.33	1.04
silicon dioxide	2.61	22.91
sodium oxide	7.72	1.18
potassium oxide	0.32	<0.03
sulfur trioxide	44.04	4.04
magnesium oxide	17.59	7.03
calcium oxide	2.53	0.27
aluminum oxide	0.94	4.53
titanium oxide	0.13	0.59
ferric oxide	5.12	8.89
vanadium pentoxide	5.82	10.9
nickel oxide	3.66	0.97
manganese oxide	0.04	0.29
lead oxide	0.67	0.94

Statistics. Data are expressed as mean values ± standard deviations. Differences between multiple groups were compared using analysis of variance (*17*). The post-hoc test employed was Scheffe's. The minimum number of animals/exposure was 3. Significance was assumed at $p < 0.05$.

Results

Gravimetric determinations demonstrated that 88.7 ± 1.2% of the dust was soluble in water (w/w; n = 6). There was little carbon in the oil fly ash, while mineral ash analysis revealed high concentrations of vanadium, nickel, and iron compounds and sulfate (Table 1). Water-soluble concentrations of vanadium, nickel, and iron in the dust accounted for 78.9%, 97.0%, and 80.4%, respectively, of the total concentrations of these metals in the oil fly ash. These metals are likely to exist as soluble sulfates and ammonium sulfates. That metal retained in the insoluble component is probably mineral oxides such as V_2O_5 and Fe_2O_3.

A neutrophilic inflammatory lung injury can result in rats after instillation of either an emission source or an ambient air pollution particle (*14*). An influx of neutrophils accounted for the majority of cells recovered by lavage after exposure of rats to the oil fly ash (Figure 1). Protein concentration in the lavage fluid was also increased, confirming an injury after exposure to oil fly ash (Figure 1). Similar to previous investigation (*16*), both the incursion of neutrophils and the increase in lavage protein concentration were associated with the soluble component of the air pollution particle (Figure 1). Relative to saline, there was no change in inflammatory cells and injury after exposure to the insoluble fraction of the oil fly ash.

Twenty-four hours after instillation of oil fly ash, prominent six-line radical adduct ESR spectra could be

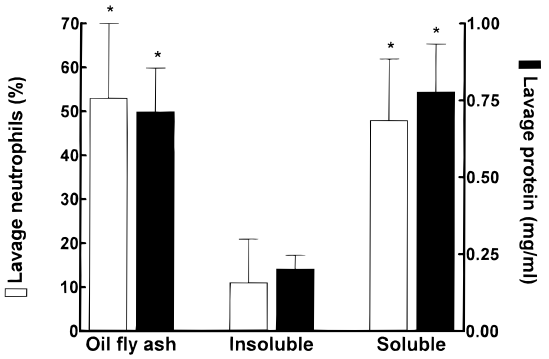

Figure 1. Percent neutrophils and protein concentrations in the lavage fluid of animals exposed to oil fly ash and its insoluble and soluble fractions. Rats were instilled with 1.0 mg of oil fly ash or the equivalent mass of insoluble and soluble components and lavaged 24 h after exposure. There was 0 ± 1% neutrophils in the lavage fluid of animals instilled with saline. Concentrations of protein in lavage fluid of animals instilled with saline were 0.125 ± 0.025 μg/mL. *Significant increase relative to instillation of saline.

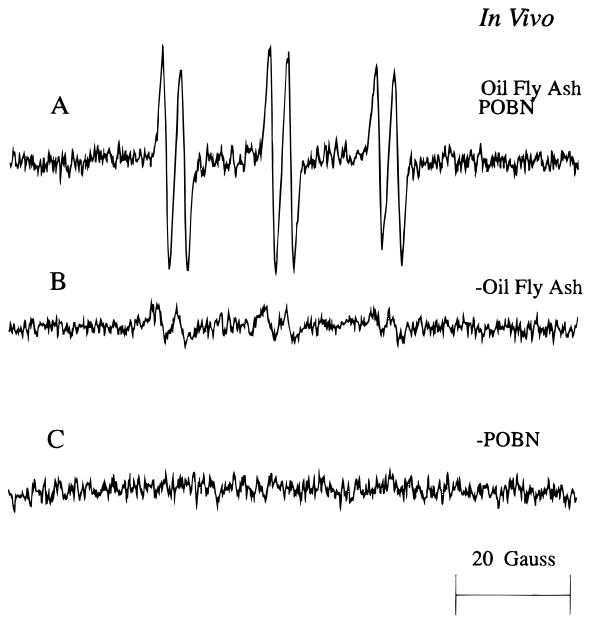

Figure 2. (A) ESR spectrum of 4-POBN radical adducts detected in lipid extracts of lungs 24 h after intratracheal instillation of oil fly ash (500 μg/rat) and 1 h after intraperitoneal administration of 4-POBN (8 mmol/kg). (B) Same as in A but rats were instilled with saline. (C) Same as in A but rats were not administered 4-POBN. Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1.33 G; time constant, 1 s; and scan rate, 5 G/min.

detected in samples of lung lipid extracts (Figure 2A). Post-hoc statistical testing demonstrated a significant increase in the intensity of the ESR spectrum after exposure to the emission source air pollution particle (Figure 3). This spectrum was simulated using a computer program developed in this laboratory (*18*). The hyperfine coupling constants for the 4-POBN radical adducts were $a^{N} = 15.0$ G and $a^{H}_{\beta} = 2.5$ G. The injection of saline resulted in a much weaker spectrum (Figure 2B). The instillation of the air pollution particle into rats without injected 4-POBN did not yield detectable radical species in lipid extracts from the lungs, confirming the dependence of radical formation on the availability of the spin trap (Figure 2C).

Lipid extracts of lungs acquired from rats exposed to the soluble component of the oil fly ash demonstrated an ESR spectrum identical to those animals instilled with

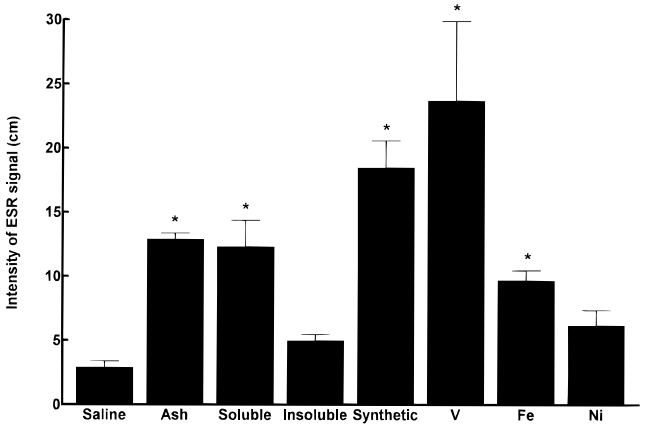

Figure 3. Intensity (mean ± standard deviation) of the ESR signals. Relative to the ESR spectrum after saline instillation, there were signficant (*) increases in the intensities of the signals after residual oil fly ash (Ash), the soluble component of the oil fly ash, the synthetic oil fly ash, vanadyl sulfate ($VOSO_4$), and ferric sulfate ($Fe_2(SO_4)_3$). However, there was no significant difference in the signal intensity of the spectra associated with exposure to the insoluble component of the oil fly ash and nickel sulfate ($NiSO_4$).

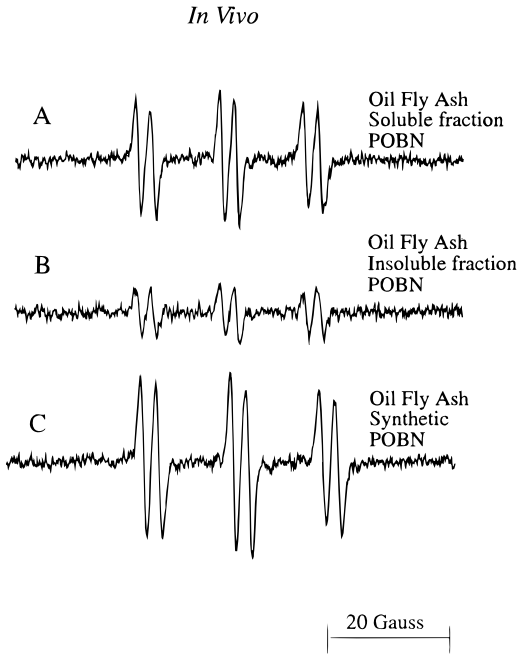

Figure 4. (A) ESR spectrum of radical adducts detected in lipid extract of lungs 24 h after intratracheal instillation of the soluble fraction of the air pollution particle (equivalent to 500 μg of oil fly ash/rat) and 1 h after intraperitoneal administration of 4-POBN (8 mmol/kg). (B) ESR spectrum of radical adducts detected in lipid extract of lungs 24 h after intratracheal instillation of the insoluble fraction of the air pollution particle (equivalent to 500 μg of oil fly ash/rat) and 1 h after intraperitoneal administration of 4-POBN (8 mmol/kg). (C) ESR spectrum of radical adducts detected in lipid extract of lungs 24 h after intratracheal instillation of a mixture of vanadyl, nickel, and iron(III) sulfates, reflecting the concentrations of these metals in the oil fly ash. Lung lipids were extracted 24 h after instillation of 500 μg of this mixture and 1 h after intraperitoneal administration of 4-POBN (8 mmol/kg). Instrumental conditions: microwave power, 20 mW; modulation amplitude, 1.33 G; time constant, 1 s; and scan rate, 5 G/min.

the air pollution particle (Figure 4A). The coupling constants were $a^{N} = 15.01$ G and $a^{H}_{\beta} = 2.46$ G. However, exposure of the animals to the insoluble component was associated with a much smaller ESR signal (Figure 4B). Post-hoc statistical testing demonstrated a significant increase in the intensity of the ESR spectrum after

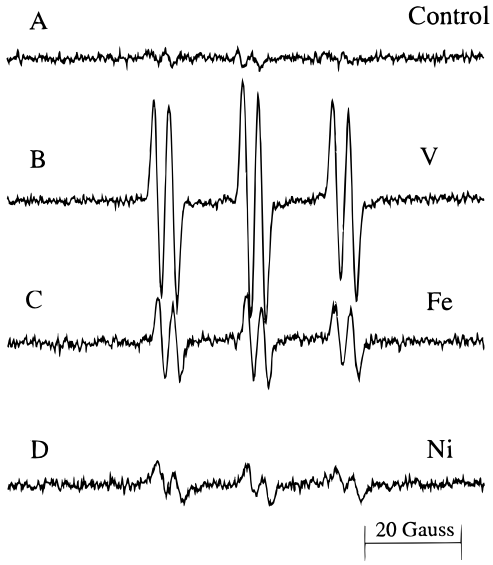

Figure 5. ESR spectrum of radical adducts detected in lipid extract of lungs 24 h after intratracheal instillation of (A) 0.5 mL of saline, (B) 0.5 mL of 1.0 mM vanadyl sulfate, (C) 0.5 mL of 1.0 mM iron(III) sulfate, and (D) 0.5 mL of 1.0 mM nickel sulfate and 1 h after intraperitoneal administration of 4-POBN (8 mmol/kg).

exposure to the soluble component only (Figure 3). The ESR spectrum of lung lipid extracts from animals instilled with a mixture of vanadyl, nickel, and iron(III) sulfates resulted in the detection of a signal comparable to that exhibited by the soluble fraction of the oil fly ash (Figure 4C).

This supported a role for the individual metal components of the particle in free radical production. Finally, individual metal sulfates (0.5 mL of 1.0 mM solutions) were instilled into the animals. Saline (Figure 5A) and nickel (Figure 5D) sulfate resulted in small ESR signals, while the spectra of lipid extract after vanadium (Figure 5B) and iron (Figure 5C) sulfates indicated a signficant production of radical adducts (Figure 3). ESR spectra after instillation of vanadyl sulfate were more intense than those acquired from animals exposed to ferric sulfate, but otherwise similar.

Discussion

ESR spectra support *in vivo* production of free radical(s) in the lungs of rats exposed to an emission source air pollution particle. The soluble component of this dust contains metal compounds and was associated with a neutrophilic lung injury. Similarly, it is this soluble fraction which appeared responsible for the production of 4-POBN adducts detected by ESR after the intratracheal instillation of oil fly ash in an animal. Instillation in a rat of both a mixture of metal sulfates, reflecting their concentrations in the oil fly ash, and the individual metal compounds included in the air pollution particle was associated with ESR spectra similar to that after exposure of the animal to the air pollution particle.

Air pollution particles have been associated with oxidant generation (*15*, *16*, *19*). Emission source particles of both natural and anthropogenic origin can include soluble metal salts and insoluble components that may have the capacity to complex metals at the surface. Metals which exist in more than one stable valence state can participate in electron transfer reactions and subsequently possess a potential to generate oxidants. Among metals which can assume two stable valence states, the first-row transition metals titanium, vanadium, chromium, manganese, iron, cobalt, nickel, and copper are found in greatest concentrations in both the crust and atmosphere of the earth. Similarly, these metals can be found in significant concentrations in air pollution particles (*20*). Exposures to these metals can be associated with oxidative stress (*21*−*24*). Soluble metal salts in greatest quantities in atmospheric particulates are likely to include acid sulfites/sulfates (*25*). Insoluble inorganic components with a capacity to complex metals at the solid interface are predominantly oxides including SiO_2, Fe_2O_3, and $A1_2O_3$. The metal compounds responsible for free radical formation associated with exposure to the oil fly ash were contained in the water-soluble fraction of this emission source air pollution particle. Vanadium, nickel, and iron sulfates are in high concentration in the oil fly ash and are included in the water-soluble fraction of the particle (*16*, *26*).

Comparable to exposure to other metal chelates (*27*), an incursion of neutrophils and a lung injury followed exposure to the oil fly ash and its soluble fraction, which included soluble metal compounds. This inflammatory injury is assumed to result from an oxidant-sensitive activation of specific promoters, including NF-κB (*28*), which signal an increase in the release of cytokines such as tumor necrosis factor and interleukins (*29*, *30*). These mediators function as chemotactic agents for inflammatory cells. Subsequently, tissue injury may directly result from exposure to an air pollution particle from either (1) the catalyzed free radicals reacting with critical molecules in the lung environment or (2) the proteases and endogenous oxidants generated by the recruited inflammatory cells.

The presentation of this ESR spectra provides evidence of an *in vivo* oxidant generation by an air pollution particle. Instillation of the soluble fraction of the oil fly ash in an animal provided an ESR spectrum similar to that of the emission source air pollution particle, while *in vitro* oxidant generation (i.e., thiobarbituric acid-reactive products of deoxyribose) supported oxygen-based free radicals as the dominant species (*14*). This suggests that metals included in the oil fly ash catalyzed oxidants (e.g., $^{\bullet}OH$) which initiated a lipid peroxidation in the lower respiratory tract of the animal. We previously reported the detection and identification of a chloroform-soluble 4-POBN radical adduct following crocidolite instillation in the rat lung. The assignment of this species to a lipid-derived radical adduct was based on the hyperfine coupling constants ($a^N = 15.01$ G and $a^H_\beta = 2.46$ G) of authentic ethyl and pentyl radical adducts of 4-POBN obtained *in vitro* (*31*). The 4-POBN radical adducts detected by ESR after instillation of the air pollution particle are very similar, if not identical, to ethyl and pentyl radical adducts. Therefore, it is proposed that in this *in vivo* system, the spin trap reacted with ethyl and pentyl radicals (or structurally similar radicals) to produce a radical adduct with hyperfine coupling constants $a^N = 15.0$ G and $a^H_\beta = 2.5$ G. Alkyl radicals are produced during the process of lipid peroxidation (*32*−*36*). These radicals result from the reductive decomposition of lipid peroxides. The carbon-centered alkyl radicals, which result from the β-scission of the lipid alkoxyl radicals, can either react with molecular oxygen to produce peroxyl radicals or abstract a hydrogen atom to form ethane and pentane, which are employed as indicators of lipid peroxidation. ESR of the lipid extract from

lungs exposed to the particle detected alkyl-type carbon-centered free radicals produced during lipid peroxidation.

Exposure to vanadium has been associated with an oxidative stress (*37*, *38*). Similar to other agents with a capacity to generate oxidants, inhalation and instillation of vanadium can induce a lung injury after inhalation (*39*). Human exposure to oil fly ash results in a lung injury in which vanadium compounds are proposed to be responsible (*40*). The intratracheal instillation of a vanadium compound produced an ESR signal comparable to those seen after exposure of the animal to oil fly ash, its soluble fraction, and the mixture of metal sulfate. These results suggest a significant role for vanadium in both oxidative stress and lung injury after exposure to an oil fly ash.

We conclude that, after instillation of an air pollution particle in the rat, ESR analysis of lung tissue demonstrates *in vivo* free radical production. This generation of free radicals appears to be associated with soluble metals in the oil fly ash.

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