Brief communication

Particulate Matter in Polluted Air May Increase Biomarkers of Inflammation in Mouse Brain

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Abstract

The etiology of neurodegenerative disorders is at present unknown. However, many of these disorders are associated with an increase in oxidative and inflammatory events. Although a small percentage of these disorders are familial cases linked to specific genetic defects, most are idiopathic. Thus, environmental factors are thought to play an important role in the onset and progression of such disorders. We have demonstrated that exposure (4 h, 5 days per week for 2 weeks) to concentrated airborne particulate matter increases inflammatory indices in brain of ovalbumin-sensitized BALB/c mice. Animals were divided into three exposure groups: filtered air (control), ultrafine particles, or fine and ultrafine particles. The levels of proinflammatory cytokines interleukin-1 alpha (IL-1α) and tumor necrosis factor alpha (TNF-α) were increased in brain tissue of mice exposed to particulate matter compared to that of control animals. Levels of the immune-related transcription factor NF-κB were also found to be substantially elevated in the brain of exposed groups compared with the control group. These data indicate that components of inhaled particulate matter may trigger a proinflammatory response in nervous tissue that could contribute to the pathophysiology of neurodegenerative diseases.
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INTRODUCTION

Epidemiological data show that particulate matter (PM) present in ambient air pollution may underlie increased morbidity and mortality rates related to pulmonary and cardiovascular systems (Pope, 2000). The increased risk of adverse health effects associated with exposure may be due to direct modulation of normal physiological function or potentiation of compromised organ responses. Histochemical analysis of small pulmonary arteries in rats treated with concentrated ambient air particles showed significant vasoconstriction in both normal and chronic bronchitis-induced animals (Batalha et al., 2002). The potentiation, of already existing impairment, by ambient particulate matter increases the vulnerability of the pulmonary system.

While adverse health effects of PM exposure on heart and lung organ systems have been extensively studied (Donaldson et al., 2001; Pope et al., 2002), relatively few studies have addressed the possibility of effects on other organs, such as the central nervous system. The focus of the present study was to determine if there is a link between PM exposure and exacerbation of events known to be present in the brain of patients with neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD).

Inflammatory parameters are chronically elevated in the aged brain (David et al., 1997; Streit et al., 1999). In

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neurodegenerative disorders, there is a further increase in such inflammation. In the brain of AD patients, reactive microglia that produce cytokines and acute phase proteins are associated with amyloid beta (Aβ) containing senile plaques, which are one of the hallmarks of the disease (Mrak et al., 1995; Styren et al., 1998). The number of activated astrocytes is increased in AD and these are associated with both senile plaques and cerebral microvessels (Cullen, 1997). Glial activation is also evident in the brain of PD patients (Banati et al., 1998). Cytokines such as IL-1, IL-6 and TNF-α are synthesized by activated microglia and macrophages in response to pathogens and trauma (Dunn, 1991). Since these are the principal proinflammatory cytokines produced by activated microglia, the level of TNF-α and IL-1α were determined in the brain tissue of mice exposed in the current study.

The potential CNS effects of ultrafine particles and the lack of studies investigating this important area of research has been previously addressed (Oberdörster and Utell, 2002). The present study demonstrates for the first time that levels of the immune-related transcription factor NF-κB, as well as the principal proinflammatory cytokines IL-1α and TNF-α, were increased in the brain after exposure to concentrated particulate matter present in air pollution. NF-κB promotes the expression of genes involved in inflammation, such as proinflammatory cytokines, iNOS, and complement factors (Baeuerle and Henkel, 1994). The importance of this protein in brain response to stress is demonstrated by studies which show that activated forms of this transcription factor were increased in experimental autoimmune encephalomyelitis (Kaltschmidt et al., 1994), prion disease (Kim et al., 1999), and aluminum exposure (Campbell et al., 2002, 2004). The reason that diverse pathogenic stimuli cause induction of NF-κB is the fundamental role it plays in the innate immune response (Hatada et al., 2000).

Since inflammatory events have been associated with neurodegenerative processes, it is possible that extended exposure to PM may aggravate events connected to the progression of these disorders.

METHODS AND MATERIALS

Animal Maintenance

Male BALB/c (6 weeks old) mice were purchased from Charles River Laboratories (Wilmington, MA) and held in an AAALAC-approved facility for 6 days before treatment. Animals were housed under barrier conditions in a vented isolation caging system (Animal Care Systems, Littleton, CO) where they were provided with PROLAB RMH 2400 lab chow (PMI Nutrition International Inc., Brentwood, MO) and water ad libitum. Mice were maintained on a 12-h light/12-h dark cycle. Protocols used in this study were approved by the Institutional Animal Use and Care Committee of the University of California, Irvine.

Antigen Sensitization, Challenge, and Exposure to Concentrated Ambient Particulates

As part of a separate study using an asthmatic mouse model (Hamelmann et al., 1999), mice were exposed for 2 weeks to concentrated ambient particulate matter in a heavily polluted urban environment. All mice were treated with daily intranasal instillation of ovalbumin in order to cause lung sensitization (Grade V, Sigma Chemical Co., St. Louis, MO; 50 μg in 20 μl of 0.9% saline). The nine mice per group were subsequently placed in individual compartments of well-characterized whole body exposure chambers (Oldham et al., 2004) sized approximately, 20 in. × 12 in. × 5 in. The animals were exposed to either purified air, concentrated airborne particulates (20-fold) less than 2.5 μm in diameter (fine plus ultrafine particulate), or concentrated airborne particulates (20-fold) less than 0.18 μm in diameter (ultrafine particulate) at a site approximately 150 m downwind of a complex of heavily trafficked highways in Los Angeles for 4 h/day, 5 days/week for 2 weeks. The prevailing winds were from the south and southwest directions with wind speeds in the 1–5 mph range, during the period of exposure.

During transport to and from the exposure location, all animals were provided purified air that had passed through activated charcoal and Purafil (buffered potassium permanganate-impregnated on activated alumina pellets; Purafil Inc., Norcross, GA) and was then HEPA-filtered to remove gaseous and particulate contaminants (Mautz and Kleinman, 1997). The control animals breathed this purified air during the exposures while the particle-challenged mice were exposed to concentrated ambient particles (CAPs). The particle concentrator is described in more detail below. The particle concentrator passes the intake aerosol through water vapor which removes water soluble gases and most reactive organic gases. The water vapor is subsequently removed with a diffusion drier. Thus, the CAPs atmosphere is largely depleted of reactive organic

and water soluble gas components as well as ozone, nitrogen oxides, and sulfur oxides. Hence, purified air was used for the controls because it better approximated the gas phase in the CAPs aerosol.

One and 2 weeks after the last exposure, mice were challenged with aerosolized ovalbumin (1%, w/v in distilled water) for 1 h to elicit an allergic response in the lungs (Hamelmann et al., 1999). The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) of the aerosolized ovalbumin were determined with an RJR (Intox, Albuquerque, NM) cascade impactor. Samples were collected during mouse exposures, and the impactor stages were extracted in distilled water. Ovalbumin extracted from the impactor stages was analyzed using a bicinchonicinic acid procedure (Bhalla and Young, 1992) with absorbance measured at 562 nm. Animals were sacrificed 1 day after the second antigen challenge.

Particle Concentrator System

Concentrated fine and ultrafine ambient particles used for the animal exposures were generated by means of concentrators developed over the past 2 years by the University of Southern California (Kim et al., 2001a, 2001b). These portable aerosol concentration enrichment systems are capable of enriching the concentration of particles by a factor of up to 22. By incorporating size-selective inlets, these systems can provide concentrated ambient particles in carefully defined size ranges. For the animal exposures, one particle concentrator used a 2.5 μm size-selective inlet (fine plus ultrafine particulate) and a second particle concentrator used a 0.18 μm size-selective inlet. In recent studies of the Southern California Supersite, the mass median diameter of elemental carbon (EC), an excellent surrogate of vehicular emissions in Los Angeles, was in the range of 0.15–0.20 μm (Kim et al., 2002). Since EC is a potentially important carrier of many toxic organic compounds and is associated with adverse health effects, we have extended the size range of the particles beyond the 0.10 μm conventional definition of ultrafine particulates to a cutoff point of 0.18 μm to ensure that we capture more of the potential toxicity of the particles.

Ambient air samples to be concentrated were drawn from outside into a duct made of aluminum to avoid particle losses due to electrostatic deposition. The concentrated aerosols were then supplied to a series of whole-body animal exposure chambers.

Physico-chemical Characteristics of Concentrated Particulate Matter

Particle flow control was affected by means of in-line calibrated rotameters (Model EW-32206-02, Cole Parmer Co., Vernon Hill, IL). Ultrafine and fine particle mass and elemental composition were measured by collecting concentrated PM on weighed 37 mm Teflon filters (PTFE 2 μm pore, Gelman Science, Ann Arbor, MI). At the end of the 10-day exposure, filters were stored at constant humidity and temperature for 24 h prior to reweighing to ensure removal of particle-bound water. Teflon filters were then analyzed by X-ray fluorescence to determine the concentrations of particle-bound metals. Samples for elemental and organic carbon constituents of PM were collected. A circular section of the quartz filter, approximately 1 cm^2, was removed from the center of the filter and subjected to thermo-analysis to determine the elemental carbon and organic carbon content. The remainder of the filter was extracted using a mixture of 0.1 ml ethanol and 5 ml of distilled deionized water and analyzed by ion chromatography to determine sulfate and nitrate content. Concentrated particles were measured continuously with a TSI 3022 Condensation Particle Counter.

Evaluation of Lipopolysaccharide (LPS, endotoxin)

Quantitative measurement of endotoxin present in the concentrated particles and ovalbumin was determined by a kinetic chromogenic *Limulus* amebocyte lysate (LAL) assay (Pyrochrome, Associates of Cape Cod, Falmouth, MA) per manufacturer's instructions. All glassware was depyrogenated at 180 °C for 4 h prior to use and all plasticware used was pyrogen free. The concentrated particulate matter samples were extracted in 10 ml of endotoxin free water (Biowhittaker Inc., Walkersville, MD) by sonication for 2 h. The samples were then centrifuged at 1400 rpm for 10 min. Sample supernatants were assayed the same day of extraction. Endotoxin was reported as endotoxin units (EU) received per mouse.

Preparation of Samples

The brains of animals were removed and quickly frozen in liquid nitrogen. Cytoplasmic and nuclear fractions were prepared using the method of Lahiri and Ge (2000). The brain tissue from each animal was weighed and homogenized in 2 ml of an ice-cold buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA,

0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and 0.5% NP-40). The samples were incubated for 10 min and centrifuged ($1500 \times g$) at 4 °C for 1 min. The supernatant containing the cytoplasmic constituents was collected and a protease inhibitor cocktail prepared by the method of Faure et al. (2001) was added. The samples were aliquoted and stored at -80 °C. The nuclear pellet was resuspended in 200 µl of a buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA and 1 mM PMSF). The samples were then centrifuged at $11,000 \times g$ for 5 min at 4 °C. The supernatant (which is the nuclear extract) was stored at -80 °C.

Electrophoretic Mobility Shift Assay

The gel shift assay was utilized to determine the extent of NF-κB activation in the nuclear fractions using a protocol developed by Promega (Madison, WI). The amount of protein in 1 µl of the extract was determined by the BCA protein assay kit (Pierce, Rockford, IL), and 75 µg of each sample, incubated with ^{32}P-labeled oligonucleotides containing the NF-κB consensus sequence, was loaded onto a gel. A negative control containing no cell extract, as well as competitor reactions were run simultaneously with the samples. The specific competitor contained unlabelled NF-κB consensus nucleotide while the nonspecific competitor contained unlabelled SP-1 consensus oligonucleotide. The competitor reactions also contained 75 µg of sample. X-ray films were manually developed. The intensity of each band was measured and quantified using an image analyzer (Eagle Eye, Strategene, San Diego, CA).

Competitive Enzyme Immunoassay

Levels of TNF-α and IL-1α were determined using a competitive enzyme immunoassay kit (Neogen Corp., Lexington, KY) designed for the detection of the total cytokine in the cytoplasmic tissue fractions. One hundred microliters of the sample, along with murine TNF-α or IL-1α antibody, and rabbit anti-mouse antibodies were added to plates precoated with the secondary goat anti-rabbit antibody. The plate was incubated at room temperature for 3 h. Murine TNF-α or IL-1α conjugate was added followed by 30 min incubation. The plate was washed and further incubated for 30 min with streptavidin-alkaline phosphatase. After another wash, 200 µl of the color reagent solution was added to the plate and the color generated was determined using a spectrophotometric plate reader set at 490 nm.

Statistical Analysis

The difference among groups was assessed using one-way analysis of variance followed by the Tukey test. Results were considered statistically significant at $p < 0.05$ using a two-tailed distribution.

RESULTS

Concentrated ambient particles in the predominantly ultrafine size range were classified as those with a mass median aerodynamic diameter of <0.18 µm (mean exposure concentration 282.5 µg/m^3) whereas the diameter of combined fine and ultrafine particles was <2.5 µm (mean exposure concentration 441.7 µg/m^3). Nearly 50% of the mass of the ultrafine particles was composed of organic carbon compounds. In contrast, the composition of the combined fine and ultrafine particles was mainly inorganic constituents (i.e., nitrates, sulfates and metals) (Table 1). For all metals analyzed in the different PM fractions, the levels were greater in the fine and ultrafine particles (Fig. 1).

The MMAD of the aerosolized ovalbumin measured using the RJR cascade impactor was 1.05 µm with a GSD of 2.1. The levels of endotoxin were determined in samples of ovalbumin used for sensitization and filters collected from the concentrated PM exposures. The data indicate that the levels of endotoxin exposure are not significantly different in the experimental groups compared with the control (Table 2).

Compared with filtered air, exposure to either ultrafine or fine and ultrafine particles significantly increased the amount of activated transcription factor NF-κB in the brain nuclear fraction (Fig. 2). This activation was not significantly different between the two particle-exposed groups. Levels of the pro-inflammatory cytokines TNF-α and IL-1α were determined in the cytoplasmic fraction of the mice brains. While the concentrations of IL-1α were increased after either particle exposure, compared with filtered air exposure,

Table 1

Chemical composition (%) of ultrafine particles or fine and ultrafine particles

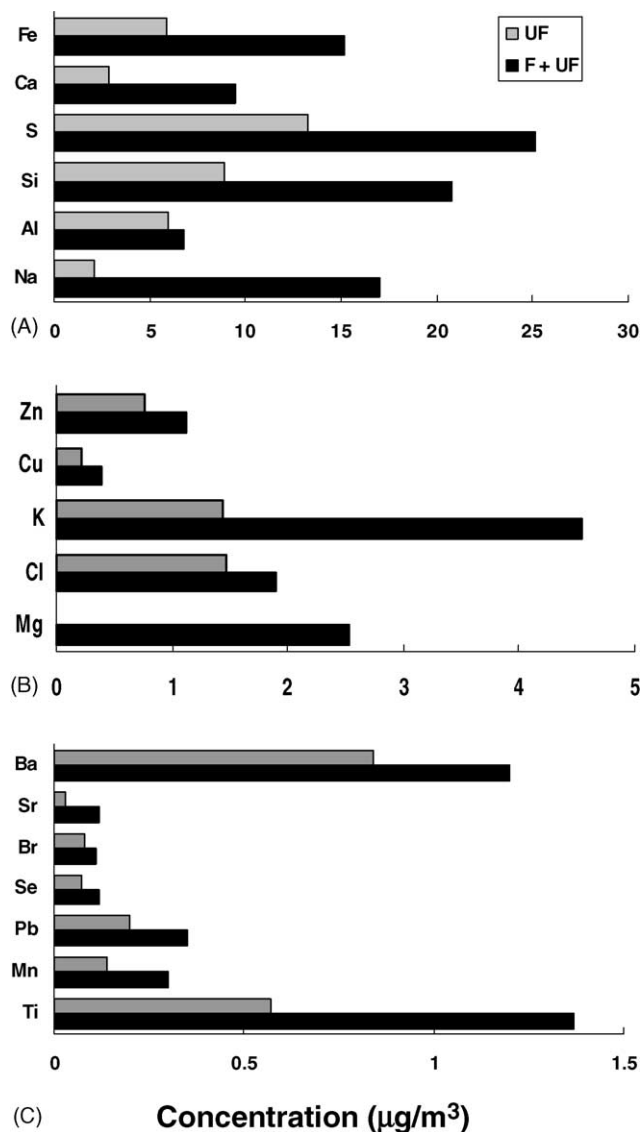| PM components	Ultrafine	Fine + ultrafine
Organic carbon	47.80	16.10
Metals	15.88	24.64
Nitrate	8.76	16.99
Sulfate	8.84	14.74
Elemental carbon	5.73	2.84

Fig. 1. Concentration of various metals in the fine + ultrafine or ultrafine PM particles. (A) Levels of the most abundantly present metals. (B) Levels of the least abundantly present metals (most common essential metals). (C) Levels of the least abundantly present metals (Other). *Abbreviations:* Fe = iron, Ca = calcium, S = sulfur, Si = silicon, Al = aluminum, Na = sodium, Zn = zinc, Cu = copper, K = potassium, Cl = chlorine, Mg = magnesium, Ba = barium, Sr = Strontium, Br = Bromine, Se = selenium, Pb = lead, Mn = manganese, Ti = titanium.

the content of TNF-α was increased only after exposure to combined fine and ultrafine particles (Figs. 3 and 4).

DISCUSSION

There is a rising concern regarding the potential adverse effects of air pollution in a multiple of organ systems. While many studies report a link between cardiopulmonary mortality and exposure to particulate

Table 2
The levels of endotoxin present in the ovalbumin used for the sensitization (total of all days), and the challenges

	EU/mouse (ng/mouse)
Sensitization	22.5 (3.7)
Exposure	
Clean air	0.011 (0.002)
F + UF	0.013 (0.002)
UF	0.014 (0.002)
Challenge	0.45 (0.007)
Boost	3.1 (0.5)
Total	26.1 (4.31)

The amount present in the different exposure settings is also indicated. The values in parenthesis are equivalent to ng of endotoxin compared to a standard of LPS.

matter in polluted air (Donaldson et al., 2001; Frampton, 2001; Pope et al., 2002), few reports have focused on the CNS. In dogs living in a highly polluted region, Southwest Metropolitan Mexico City (SWMMC), an increase in brain inflammation was documented when compared with animals which lived under less polluted conditions (Tlaxcala, Mexico). The cortical tissue of animals from SWMMC had higher levels of NF-κB activation, iNOS production and reactive astrocytes compared with the animals from Tlaxcala (Calderon-Garciduenas et al., 2002). However, in this study, the dogs were of mixed breeds and the exposure conditions and diets were not standardized. Furthermore, the age of the animals was variable (ranging from 1 day to 12 years old), and inflammatory events
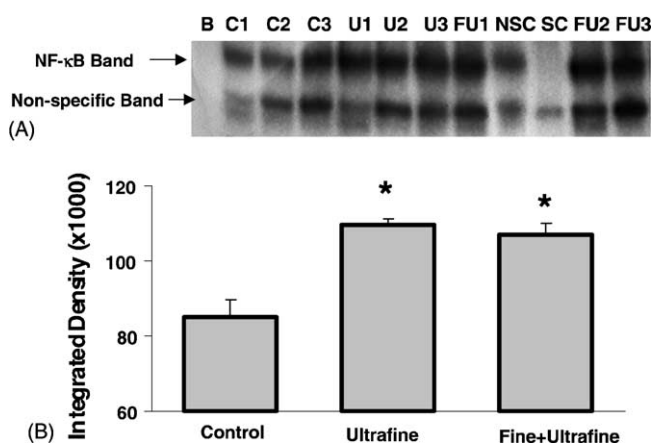

Fig. 2. Gel shift mobility analysis of levels of activated NF-κB in cortical tissue (A). Typical gel shift showing three separate samples per group (C = control, U = ultrafine particles, FU = fine + ultrafine particles, SC = specific competitor containing sample and unlabelled NF-κB consensus oligonucleotide, NSC = non-specific competitor containing sample and unlabelled SP1 consensus oligonucleotide) (B). Integrated density of the shifted band. *Value is significantly different ($p < 0.05$) from the control. Bars represent mean of nine individual animals ± S.E.
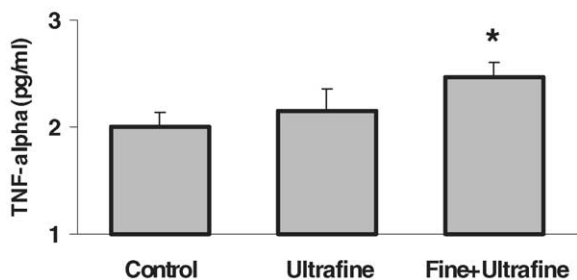
Fig. 3. Levels of TNF-α in the cytoplasmic fraction of the brain after exposure to particulate matter. *Value is significantly different ($p < 0.05$) from the control. Bars represent mean of nine individual animals $\pm$ S.E.

are known to increase with senescence (Streit et al., 1999). The air pollution in SWMMC is a complex mixture of mainly ozone, PM_{10}, and aldehydes. It is difficult to determine which component was causing the greatest effect. Such preponderance of confounding factors makes it difficult to conclude that the changes observed were due to PM exposure alone. In another study, levels of glial fibrillary acidic protein (GFAP), a marker of astrocyte proliferation, were unaltered in the brain of rats exposed for 13 weeks to combustion products derived from the burning of 100% soybean oil (Finch et al., 2002).

The present report reveals for the first time that there is a significant increase in levels of several inflammatory markers in the brain of mice exposed to PM in a standardized, relatively controlled setting. Since mediators are constitutively expressed by the innate immune system present in nearly all mammalian organs, this is not surprising. There was a baseline level of proinflammatory mediators in the brain of control animals and this may have been due to the nasally administered ovalbumin used for a model of lung sensitization. However, this affected both the control and exposed mice to the same degree and is unlikely to explain the PM-induced increase in inflammatory mediators. Furthermore, the effects observed
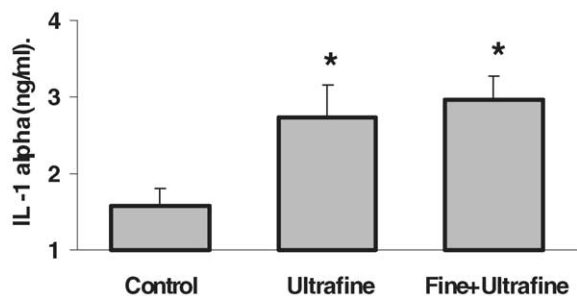

Fig. 4. Levels of IL-1α in the cytoplasmic fraction of the brain after exposure to particulate matter. *Value is significantly different ($p < 0.05$) from the control. Bars represent mean of nine individual animals $\pm$ S.E.

were probably not due to increased levels of endotoxin in the PM. The amount of activated transcription factor NF-κB was greater in the brain tissue of PM-exposed animals compared with controls. Levels of IL-1α in the brains of treated mice were also enhanced. TNF-α was significantly elevated in the brains of animals treated with fine and ultrafine particles. In addition, the levels of this cytokine were increased in the brains of animals exposed to ultrafine particles alone. Since metals constitute a high proportion of the fine particle constituents, it is possible that metals may account for the greater TNF-α response following exposure to this fraction. However, to ensure the validity of this conclusion, more detailed studies on the effect of inhaled metal and brain inflammation need to be conducted. This is an important area of research because there is evidence of metal involvement in the pathology of neurodegenerative disorders.

Abnormal metal interactions have been indicated in the pathogenesis of AD (Bush, 2003) and the levels of copper, zinc and iron are increased in the rims of senile plaques (Lovell et al., 1998). Iron is also elevated in the brain of PD patients (Hirsch et al., 1991). Our results show airborne levels of these metals as constituents of PM. These may directly reach the CNS through the olfactory pathway via cell processes of olfactory neurons that enter the brain through the cribriform plate of the ethmoid bone. For inhaled manganese, the olfactory route has been shown to contribute significantly to the access of this metal into the brain (Brenneman et al., 2000). However, more experimental studies should be conducted to determine if this pathway is indeed important for the entry of constituents of PM into the CNS and causation of a proinflammatory response. It is possible that the CNS effects observed in our study are primarily due to systemic soluble inflammatory mediators crossing the blood brain barrier.

The extent to which induction of inflammatory parameters in the brain of PM-exposed animals may lead to potentially adverse consequences is at present unknown. As reviewed by Eikelenboom and van Gool (2004), activated microglia in the vicinity of amyloid plaques are immunoreactive for the proinflammatory cytokines (IL-1, IL-6 and TNF-α). Chronic administration of the classical pro-inflammogen LPS into the fourth ventricle of rats caused astrogliosis and an increase in the mRNA for IL-1β, TNF-α, amyloid precursor protein (APP) and glial fibrillary acidic protein (GFAP). This was followed by hippocampal cell loss and impairment of spatial memory (Haus-Wegrzyniak et al., 1998). These changes mirror abnormalities found in the AD brain. Furthermore,

levels of TNF-α in the cerebrospinal fluid of patients with 'mild cognitive impairment', who after nine months progressed to AD, are significantly higher than those of controls (Tarkowski et al., 2003). This study brings into question whether an increase in proinflammatory cytokine levels in the brain may accelerate AD progression in predisposed individuals. In rats, LPS treatment also led to microglial activation followed by selective loss of dopaminergic neurons, a hallmark of PD (Castano et al., 1998; Gao et al., 2002). It is possible that analogous to the effects observed with LPS-induced CNS inflammation, chronic exposure to PM present in air pollution may also lead to exacerbation of events associated with neurodegeneration.

Since the etiology of a major fraction of neurodegenerative cases is idiopathic, it is important to evaluate the role of environmental air pollution as a possible causative factor in exacerbation of these disorders. The findings presented here suggest that environmental exposure to particulate matter may indeed enhance events in the central nervous system connected to neurodegenerative processes. However, the animals in this study represent an asthmatic model, sensitized intranasally with ovalbumin. Further studies need to determine whether the proinflammatory effects observed will be present in non-sensitized animals, providing a more physiologically relevant model for evaluating exposure to PM.

PM present in air pollution may contribute to the progression of pre-existing changes relating to the pathology of age-related neurological diseases. The neuroanatomical loci of the inflammatory changes remain to be elucidated. More epidemiological and mechanistic studies on the effects of inhaled particulate matter on the brain are warranted before concluding that exposure to PM increases inflammatory processes in the brain and that this may be relevant to the progression of neurodegenerative diseases. The use of transgenic animal models of age-related neurological disorders will be important in evaluating whether PM exposure can exacerbate formation of pathological lesions characteristic of neurodegenerative disease processes.

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