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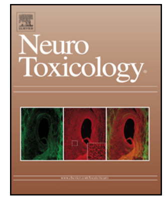

Particulate matter neurotoxicity in culture is size-dependent

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ABSTRACT

Exposure to particulate matter (PM) air pollution produces inflammatory damage to the cardiopulmonary system. This toxicity appears to be inversely related to the size of the PM particles, with the ultrafine particle being more inflammatory than larger sizes. Exposure to PM has more recently been associated with neurotoxicity. This study examines if the size-dependent toxicity reported in cardiopulmonary systems also occurs in neural targets. For this study, PM ambient air was collected over a 2 week period from Sterling Forest State Park (Tuxedo, New York) and its particulates sized as Accumulation Mode, Fine (AMF) (>0.18–1 μm) or Ultrafine (UF) (<0.18 μm) samples. Rat dopaminergic neurons (N27) were exposed to suspensions of each PM fraction (0, 12.5, 25, 50 μm/ml) and cell loss (as measured by Hoechst nuclear stain) measured after 24 h exposure. Neuronal loss occurred in response to all tested concentrations of UF (>12.5 μg/ml) but was only significant at the highest concentration of AMF (50 μg/ml). To examine if PM size-dependent neurotoxicity was retained in the presence of other cell types, dissociated brain cultures of embryonic rat striatum were exposed to AMF (80 μg/ml) or UF (8.0 μg/ml). After 24 h exposure, a significant increase of reactive nitrogen species (nitrite) and morphology suggestive of apoptosis occurred in both treatment groups. However, morphometric analysis of neuron specific enolase staining indicated that only the UF exposure produced significant neuronal loss, relative to controls. Together, these data suggest that the inverse relationship between size and toxicity reported in cardiopulmonary systems occurs in cultures of isolated dopaminergic neurons and in primary cultures of the rat striatum.

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1. Introduction

Particulate matter (PM) has been epidemiologically associated with death and cardiopulmonary illnesses in the elderly and in those individuals with pre-existing respiratory conditions (e.g., smokers, asthmatics) (Brook et al., 2010; Krewski et al., 2009; Pope and Dockery, 2006). Numerous animal studies indicate that inhalation of PM results in inflammatory toxicity in the airways and cardiovascular effects (Chen et al., 2010; deHaar et al., 2006; Maier et al., 2008; Oberdorster et al., 2000). Although the historical focus of PM toxicity has been on these cardiopulmonary targets, it is now appreciated that inhaled nano-size particulates can quickly exit the lungs and enter the circulation where they distribute (i.e., liver, kidneys, testes, lymph nodes, brain) (Kreyling et al., 2004; Oberdorster et al., 2002) and these organ systems through oxidative stress (Pohjola et al., 2003; Samet et al., 2004; Schulz, 2006).

Because of its high energy demands and its inherently low level of endogenous scavengers (e.g., vitamin C, catalase, superoxide dismutase) the brain is extremely sensitive to free radical damage (Halliwell, 1992; Tanaka, 1997). In the central nervous system (CNS), oxidative stress is largely mediated by microglia, which are macrophage-like, phagocytic cells that are activated by a broad range of stimuli, including PM (Block et al., 2004; Sama et al., 2007) and nanoparticles (Long et al., 2006). Once activated, the microglia produce multiple reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radicals and peroxy-nitrites (Block et al., 2007; Fariss et al., 2005) that can diffuse from their plasma membrane and damage nearby neurons. Dopaminergic neurons are especially vulnerable to oxidative stress damage since they support a high synaptic network within the mesencephalic brain (striatum) and because dopamine metabolism in itself generates ROS (Double et al., 2010; Gao et al., 2002; Gonzalez-Hernandez et al., 2010; Jackson-Lewis and Smeyne, 2005; Mitchell et al., 1999). The striatum is a brain area, which houses large populations of dopaminergic neurons and a disproportionately large population of microglia (Hirsch, 1994; Koutsilieri et al., 2002).

The major components of PM include transition metals, sulfate and nitrate ions, organics, minerals, adsorbed gases, and biocontaminants (e.g., endotoxins, mold, pollen), attached to a core of carbonaceous material. The size of the particulates found in PM has been inversely related to its inflammatory damage (deHaar et al., 2006; Oberdorster, 1996; Pedata et al., 2010; Valavanidis et al.,

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2008), with smaller, ultrafine size particles being more toxic to the target tissue. Mechanisms to explain this phenomenon may be physiological and/or physicochemical in nature. The current study examines if this phenomenon (size-dependent toxicity) can be reproduced in isolated dopaminergic neurons (N27) and cultures dissociated from the embryonic rat brain striatum sized-PM material.

2. Materials and methods

2.1. Collection of size-fractionated ambient PM samples

The PM samples were collected over a two-week period in August 2005, within the Sterling Forest State Park (Tuxedo, NY). The park is a largely undeveloped woodland park located approximately 50 km north-west of Manhattan. It is bordered by a lightly traveled two-lane road and there are no large power generators or industrial operations within 20 miles of the collection site. The ambient air in Sterling Forest is considered as regional background PM and is representative of the ambient air over the Virginia-Maine megalopolis (Maciejczyk and Chen, 2005). The air samples were collected using the Multi-Orifice Uniform Deposit Impactor (MOUDI) (MSP, St. Paul, MN) which size-classifies the PM material as it is collected. Sized-samples were classified as Accumulation Mode Fine (AMF) (>0.18–1 μm) or Ultrafine (UF) (<0.18 μm). The ambient air in Sterling Forest is considered as regional background PM and is representative of the ambient air over the Virginia-Maine megalopolis. The volume-size distribution of ambient air collected in this area of the park during the spring and summer is bimodal with mean diameters in both the AMF and UF range. The volume-size distribution of ambient air collected from Sterling Forest are described in detail in (Maciejczyk et al., 2005).

Samples were collected on pre-weighed Teflon filters and then immersed in Dubelco's Minimal Essential Media to yield a concentration of 500 μg/ml. Aliquots of these suspensions were diluted to stock concentrations for in vitro exposure. Care was taken not to sonicate or vigorously vortex the suspensions to preserve their original size at the time of collection.

Analyses for 35 elements were performed New York University's A. J. Lanza Laboratory by non-destructive XRF (ModelEX-6600-AF; Jordan Valley) using five secondary fluorescers (Si, Ti, Fe, Ge, and Mo), and spectral software XRF2000v3.1 (US EPA and ManTech Environmental Technology). The major components in the test samples consisted of sulfur (most likely as secondary aerosol particles) and elements associated with soil (e.g., Si, Al, Ca, and Fe) (data not shown). A detailed composition analysis of the ambient samples collected at Sterling Forest has been reported previously (Maciejczyk and Chen, 2005).

2.2. Cells and culture maintenance

The PM samples were tested on N27 neurons, an immortalized dopaminergic immortalized neuron from the mesencephalic brain of wild type, Sprague–Dawley rats (Zhou et al., 2000). N27 neurons were grown in RPMI 1640 media, supplemented with 10% fetal calf serum and 1% penicillin–streptomycin (ATCC, Manassas, VA). The N27 neurons were plated in 96 well plates (Costar, Corning, Inc., Corning, NY) and grown to ~85% confluency before exposure to the individual particulate samples. All cell culture reagents were purchased from InVitrogen, Carlsbad, CA.

Primary cultures of Sprague–Dawley embryonic striatum were purchased from BrainBits^{TM} (http://www.brainbitsllc.com). Upon receipt the tissues were triturated in Neurobasal^{TM}, a serum-free media supplemented with glutamine, plated on poly-lysine-coated BTM 96 well plates (Nagle Nunc International-NUNC^{TM}, Rochester, NY), and grown to 85% confluency before exposure.

2.3. Neuronal assays

To examine if PM was neurotoxic to isolated neurons, N27 cells were exposed to non-sonicated aliquots of AMF and UF (12.5, 25, and 50 μg/ml) for 24 h. After exposure, cells were washed multiple times to remove extraneous particles. Neuronal loss was measured with Hoechst 33258, a nuclear stain that binds to the adenine–thymine rich regions of double stranded nuclear DNA. After exposure, cells were washed with Hank's basic salt solution, and then lysed by alternating temperatures (1 h at 37 °C followed by 1 h −80 °C). Hoechst reagent (2.0 μg/ml) was added to each well for 30 min and the plates spectrophotometrically read at 346/460 nm wavelengths.

Reactive nitrogen species and nitric oxide are important physiological messengers in neuronal and cardiovascular tissues (Halliwell, 1992; Mohanakumar et al., 2002). Inducible nitric oxide is generated from microglia (Chandra et al., 2000; Fariss et al., 2005) and can be measured with nitrite, a primary and stable breakdown product of nitrous oxide (Hengartner, 2000). Nitrite was measured in complex brain (striatum) cultures using Griess reagent under acidic (pH 4.0) conditions (Miranda et al., 2001). Plates were read using chemiluminescence.

For all spectrophotometric measurements, cells were plated in either black (fluorescence) or white (chemiluminescence) 96-well culture plates (Corning Inc., Corning, NY) to minimize light scatter. Spectrophotometers (Molecular Device, Sunnyvale, CA) included the Spectramax EM (fluorescence) and Lmax II 384 (chemiluminescence) plate readers.

2.4. Immunohistochemistry

Neurons in the striatum cultures were selectively stained with (mouse, rat, human) neuron specific enolase (NSE) (Dako Inc., Glostrup, Denmark, www.dako.com) at a 1:200 concentration according to protocol. For visualization, cells were biotinylated, linked to Streptavidin and counter-stained with a chromagen substrate according to directions given in the LSAB 2 System-HRP (Dako Inc.). Support reagents were purchased from Sigma (St. Louis, MO).

2.5. Morphometry

Semi-confluent cultures ($n = 3$ wells) of embryonic brain striatum were exposed to AMF (80 μg/ml) or UF (8 μg/ml) for 24 h. After exposure, cultures were fixed in 3.7% formalin, immunocytochemically stained, and analyzed morphometrically for neuronal loss. For this, six photographs were taken of each well (10× apocromatic lens) using a Nikon TE300 inverted microscope fitted with a cooled-frame CCD camera (Orca I, Hamamatsu, Inc.). Each digitized image was analyzed using Metamorph 7.0 software (Molecular Device, Sunnyvale, CA). Threshold levels of pixel stain were used to quantitate the Total Area (i.e., neuronal cell bodies and axonal plexus) of NSE staining and the values binned according to area. Shape and size parameters were also defined to describe the neuronal cell body alone and applied to the Total Area data set using the Integrated Morphometric Analysis mode. Values for the Total Area and those measuring the neuronal cell bodies were collected in AMF (80 μg/ml and UF (8 μg/ml) or) treated cultures.

2.6. Statistical analysis

All data were collected using SoftMax Pro 4.8 software (Molecular Devices, Sunnyvale, CA). Graphing was performed with GraphPad Prism 4.02. Data were analyzed using a one-way ANOVA with Dunnett's post-testing to determine the lowest

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