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Macrophages treated with particulate matter PM_{2.5} induce selective neurotoxicity through glutaminase-mediated glutamate generation

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Abstract

Exposure to atmospheric particulate matter PM_{2.5} (aerodynamic diameter 2.5 µm) has been epidemiologically associated with respiratory illnesses. However, recent data have suggested that PM_{2.5} is able to infiltrate into circulation and elicit a systemic inflammatory response. Potential adverse effects of air pollutants to the central nervous system (CNS) have raised concerns, but whether PM_{2.5} causes neurotoxicity is unclear. In the present study, we have demonstrated that PM_{2.5} impairs the tight junction of endothelial cells and increases permeability and monocyte transmigration across endothelial monolayer *in vitro*, indicating that PM_{2.5} is able to disrupt blood-brain barrier integrity and gain access to the CNS. Exposure of primary neuronal cultures to PM_{2.5} resulted in decrease of cell viability and loss of neuronal antigens. Furthermore, supernatants collected from PM_{2.5}-treated macrophages and microglia were also neurotoxic. These macrophages and microglia significantly increased extracellular levels of glutamate following PM_{2.5} exposure, which were negatively correlated with neuronal viability. Pre-treatment with N-Methyl-D-aspartate (NMDA) receptor antagonist MK801 alleviated neuron loss, suggesting that PM_{2.5} neurotoxicity is mediated by glutamate. To determine the potential source of excess glutamate production, we investigated glutaminase, the main enzyme for glutamate generation. Glutaminase was reduced in PM_{2.5}-treated macrophages and increased in extracellular vesicles,

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suggesting that PM_{2.5} induces glutaminase release through extracellular vesicles. In conclusion, these findings indicate PM_{2.5} as a potential neurotoxic factor, crucial to understanding the effects of air pollution on the CNS.

Keywords

PM_{2.5}; blood-brain barrier; macrophage; glutamate; glutaminase; neurotoxicity

Introduction

Particulate matter (PM) is a mixture of solid particles and liquid droplets suspended in air. Based on the sizes and aerodynamic properties, PM can be classified as PM₁₀ (aerodynamic diameter $\geq 10 \mu\text{m}$), PM_{2.5} (aerodynamic diameter $\leq 2.5 \mu\text{m}$) and ultrafine particulate matter (UFP, aerodynamic diameter $\leq 0.1 \mu\text{m}$) (Block & Calderón-Garcidueñas 2009). The prevalence of these different forms of PM has caused an ongoing environmental crisis in many parts of the developing world that affects public health. Although a majority of PM research focuses on lung and cardiopulmonary targets, it is increasingly likely that PM may affect tissues beyond the respiratory system (Wagner *et al.* 2014, Liu *et al.* 2014, Hussain *et al.* 2014, Vora *et al.* 2014).

The possibility that the central nervous system (CNS) might also be targeted by PM was first proposed in a 2002 editorial (Oberdorster & Utell 2002). Suspected fractions of PM implicated in the adverse CNS effects include PM_{2.5} and UFP, both of which are inhaled and cross the blood-air barrier of the lungs, gaining access to the circulatory system. Nanosized particles can cross the blood-brain barrier (BBB) and physically enter human (Block & Calderón-Garcidueñas 2009) and animal brains (MohanKumar *et al.* 2008, Bondy 2011). Indeed, recent data have suggested that PM induces neuroinflammation and microglia activation by increasing the brain levels of activated nuclear factor kappa B (NF- κ B) and the pro-inflammatory cytokines, especially tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) (MohanKumar *et al.* 2008, Levesque *et al.* 2011). However, it is unclear which fraction of the PM mediates the neuroinflammatory effects.

Glutamate is produced by various cells in the brain and is released by neurons as the main excitatory neurotransmitter. In the brain, the extracellular concentrations of glutamate are deliberately kept low to prevent overstimulation of post-synaptic cells. The removal of glutamate is typically maintained by astrocytes (Pellerin & Magistretti 1994, Storck *et al.* 1992). However, persistently elevated extracellular glutamate, produced by macrophages (MP) as a consequence of viral infection and immune activation, can induce neuronal injury (Zhao *et al.* 2004). In the CNS, mitochondria glutaminase catalyzes the deamination of glutamine into glutamate and is the primary enzyme responsible for the generation of glutamate (Zhao *et al.* 2004, Huang *et al.* 2011). Two glutaminase (GLS) isozymes exist, including GLS1 and GLS2. Furthermore, two GLS1 allozymes, glutaminase C (GAC) and kidney-type glutaminase (KGA), are both present in the brain (Holcomb *et al.* 2000, Huang *et al.* 2011). Interestingly, IL-1 β or TNF- α treatment increased the levels of KGA in cytosol and TNF- α specifically increased KGA levels in the extracellular fluid, away from its main

residence in mitochondria (Ye *et al.* 2013). This released enzyme is capable of rapidly converting the abundant extracellular amino acid glutamine into excitotoxic levels of glutamate in an energetically favorable process (Erdmann *et al.* 2009). Extracellular vesicles are shedding vesicles derived from many different cell types under both normal and pathological conditions in the brain (Skog *et al.* 2008). Recently, we have demonstrated that extracellular vesicles mediate glutaminase release in HIV-1-infected and immune activated macrophages (Wu *et al.* manuscript in preparation).

To determine whether PM_{2.5} induces neurotoxicity and the mechanism involved, we first determined the effects of PM_{2.5} on endothelial monolayer and found that PM_{2.5} disrupted and increased endothelial monolayer permeability, permitting PM_{2.5} to interact directly with CNS cell types. We then determined neurotoxicity and examined the glutamate production in macrophages after PM_{2.5} exposure. Furthermore, we targeted the regulation of glutaminase and identified extracellular vesicles-mediated glutaminase release as a possible cause of excess levels of glutamate after PM_{2.5} exposure.

Materials and methods

PM_{2.5} sampling and preparation

Ambient PM_{2.5} was collected onto Teflon filters (diameter = 47 mm, Whatman, USA) using low volume samplers (42 L/min, URG, USA) at Yuquan Road, Beijing, China (September – December, 2012). The preparation of PM_{2.5} was performed as Imrich described (Imrich 2000). Briefly, Teflon filters were suspended in ultra-pure water (18.2 MΩ/cm) and probe sonicated for 1 min. Suspensions of PM_{2.5} were prepared in ultra-pure water at 5 mg/ml. All filters were equilibrated for over 48 h in a humidity (30%) and temperature (25°C) controlled room. To determine the mass of PM_{2.5}, we weighed both the blank filters and PM_{2.5} filters on a high-precision microbalance (Mettler Toledo, AG258, OH, USA).

Exposure of cells to PM_{2.5}

Cells were exposed to freshly dispersed PM_{2.5} preparations at final concentrations of 6.25, 12.5, 25, 50 or 100 µg/ml for 24 h. These concentrations were chosen based on the exposure estimate in the published literatures (Baccarelli *et al.* 2014, Sun *et al.* 2001, Oberdörster *et al.* 2002). For combination treatment of cells with bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES, a glutaminase inhibitor) and PM_{2.5}, BPTES was added to the culture at a final concentration of 1 µM along with PM_{2.5} for 24 h. For combination treatment of cells with MK801 (NMDA receptor antagonist) and PM_{2.5}, MK801 was added to the culture at a final concentration of 3 µM for 2 h before the PM_{2.5} treatment. Equal volumes of ultra-pure water were used in the mock-treated samples as vehicle controls for PM_{2.5}.

Isolation and culture of primary human neurons and rat cortical neurons

Human neurons were prepared from human fetal brain tissue (gestational age 13–16 weeks), which was obtained from elective abortions in full compliance with the University of Nebraska Medical Center (UNMC) and NIH ethical guidelines. Primary rat cortical neurons (RCN) were prepared from cortices of embryonic day 17 to 18 (E17-18) Sprague-Dawley rat

fetuses (Charles River Laboratories International Inc., Wilmington, MA). Both human neurons and RCN were isolated and cultured as previously described (Ye *et al.* 2013, Tian *et al.* 2012). Neurons were plated on poly-D-lysine (Sigma-Aldrich, St. Louis, MO) coated 48-well plates and cultured in neurobasal media (Invitrogen) supplemented with B27 (Invitrogen), 0.5 mM glutamine and 1% penicillin/streptomycin (Sigma-Aldrich). Human neurons and RCN were deemed mature and used for experiments at 14 and 7 days after initiation of *in vitro* cultures, respectively.

Cultures of primary monocyte-derived macrophages and human microglia

Human monocytes were isolated from peripheral blood mononuclear cells. Monocytes were plated on 24-well plates at a density of 1.1×10^6 cells/well and differentiated for 7 days in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% heat-inactivated pooled human serum, 10% macrophage colony stimulating factor (M-CSF, UNMC tissue core), 50 µg/ml gentamicin (Sigma-Aldrich) and 10 µg/ml ciprofloxacin (Sigma-Aldrich). Human fetal microglia culture was prepared from fetal brain tissue-derived microglia-astrocytes mixed culture as previously described (Ghorpade *et al.* 1998). The cells were cultured in DMEM (GIBCO) supplemented with M-CSF and 10% fetal bovine serum (Atlanta Biologicals) for an additional 7 days before the experiments.

Cultures of human umbilical vein endothelial cells (HUVEC) and human brain microvascular endothelial cells (HBMEC)

Human umbilical vein endothelial cells (HUVEC, ATCC® PCS-100-010™) were cultured in Vascular Cell Basal Medium supplemented with Endothelial Cell Growth Kit-VEGF (ATCC). Human brain microvascular endothelial cells (HBMEC) were primary cell cultures derived from surgically removed brain tissues, which were acquired from Dr. Maria Brito's laboratory (Bernas *et al.* 2010). The cultures are maintained in 1 mg/ml heparin, and 1% penicillin/streptomycin (Sigma-Aldrich). The determination of the tight junctions (TJs) protein (zonula occludens protein-1, ZO-1; zonula occludens protein-2, ZO-2) in HUVEC and HBMEC after PM_{2.5} exposure and the use of HUVEC to model BBB were performed as previously described (Germann *et al.* 2008).

Ethics statement

Primary RCN were prepared in accordance with ethical guidelines for care and use of laboratory animals set forth by the National Institutes of Health (NIH), with Institutional Animal Care and Use Committee (IACUC) #: 04-097-01; Monocytes were used in full compliance with the University of Nebraska Medical Center and NIH ethical guidelines, with the Institutional Review Board (IRB) #: 123-02-FB. We have the informed written consent from all participants involved in this study.

MTT reduction assay

Cell viability was assessed by methyl thiazolyl tetrazolium (MTT) reduction. Briefly, cells were incubated with 100 µl of 10% MTT solution in culture media for 1 h at 37°C. Equal volume of dimethylsulfoxide (DMSO) was added to dissolve the formazan converted from

MTT by mitochondrial dehydrogenase. After 15 min incubation, cell viability was determined by measuring absorbance at 490 nm using a microplate reader.

Cell and TJs permeability

Cell and TJs permeability was tested as previously described (Yao *et al.* 2011). Briefly, HUVEC at passages 6–8 were seeded on 6.5-mm polyester Transwell inserts (0.4- μ m pore size) (Fisher Scientific, Pittsburgh, PA) and grown for 7 d to confluence. After confluency, HUVEC monolayers were exposed to PM_{2.5} (25 μ g/ml) for 24 h. To detect changes in monolayer permeability, fluorescein isothiocyanate Dextran-4 (FITC Dextran-4, 1 mg/ml; average mol wt 4,000, Sigma-Aldrich) was added to the upper chamber of the Transwell plates. The monolayer-free upper chamber group was set as the positive control. Culture medium without FITC Dextran-4 was set as blank. Medium (100 μ l) from the lower chamber were collected for fluorescent measurement using excitation and emission wavelengths of 488 and 530 nm, respectively.

Monocyte migration

Monocyte migration assay was performed as Persidsky described (Persidsky *et al.* 1997). Briefly, a single monolayer of HUVEC was produced by placing 200 μ l of cell suspension containing 5×10^5 cells/well in the upper chamber of a 24-well tissue culture insert (3 μ m pore size) (Fisher Scientific) and 1 ml of culture medium in the lower chamber. After 7 d culture, the monolayer grew to confluence. HUVEC monolayers were exposed to PM_{2.5} (0, 6.25, 12.5, 25, 50 or 100 μ g/ml) for 24 h. Monocytes (5×10^5 cells/well in 200 μ l) were loaded on the upper chambers of the transwells. The migration process proceeded for 12 h. Coverslips were loaded on the bottom of the lower chambers. The number of migrated monocytes was determined by diamidino-phenyl-indole (DAPI) staining.

Immunofluorescence

Primary RCN cultures were plated in poly-L-lysine-coated 48-well plates at concentrations of 1×10^5 cells/well. After 7-d in culture, cells were treated with PM_{2.5} or macrophage-conditioned media (MCM) for additional 24 h. Human monocytes were plated on 24-well plates at a density of 1.1×10^6 cells/well and differentiated for 7 days to become macrophages. Cells were fixed with 4% paraformaldehyde (PFA) for 15 min and penetrated with 0.1% triton-X 100 for another 15 min. Fixed RCN and monocyte-derived macrophages were blocked with 5% BSA (Invitrogen) in PBS and incubated for 1 h with antibodies against MAP2 (Millipore) or CD68 (Dako) respectively, followed by incubation with goat anti-mouse IgG (H+L) Alexa Fluor 568 (Invitrogen) for 1 h. Nuclei were stained by DAPI (Sigma-Aldrich). MAP2 and CD68 staining were examined by a Nikon Eclipse TE2000E fluorescent microscope and photographed by a digital camera (CoolSNAP EZ, Photometrics). Quantification of MAP2 staining was performed by Image-ProPlus, version 7.0 (Media Cybernetics). The assessors were blinded during image acquisition or quantification. Bright field images of microglia were captured with the same Nikon microscope and camera under the dia-illumination.

Western blot

Proteins were isolated with mammalian Protein Extraction Buffer (Pierce) containing a protease inhibitors cocktail (Roche Diagnostics). The lysates were centrifuged at 12,000 g for 10 min at 4°C, and then protein concentration was determined with BCA Protein Assay Kit (Pierce). Equivalent samples (20 µg protein) were subjected to sodium dodecyl sulfate–poly-acrylamide gel electrophoresis on 10% or 15% gel. After electrophoretic transfer to polyvinylidene difluoride membranes (Millipore and Bio-Rad), proteins were probed with caspase 3 (Cell Signaling Technology), ZO-1 and ZO-2 (Cell Signaling Technology), KGA and GAC (Dr. N. Curthoys, Colorado State University, Fort Collins, CO), or β -actin (Sigma-Aldrich) antibodies overnight at 4°C followed by a horseradish peroxidase-linked secondary anti-rabbit or anti-mouse antibody (Cell Signaling Technology). Antigen–antibody complexes were visualized by Pierce ECL Western Blotting Substrate (Pierce).

Analysis of glutamate by HPLC

High performance liquid chromatography (HPLC) analysis was performed using an Agilent 1200 liquid chromatograph and fluorescence detector as previously described (Huang *et al.* 2011). Briefly, each 5 µl sample or standard solution was derived with ortho-phthalaldehyde (Agilent) for fluorescence detection. For each experiment, 250 µl of sample was mixed with equal volumes of 3% perchloric acid (Aldrich). This mixture was then immediately neutralized with 11.5 µl of saturated potassium carbonate (Aldrich). Samples were centrifuged at 14,000 rpm for 15 min at 4°C. Samples (60 µl) were then injected into the HPLC system. Glutamate detection was measured using a fluorescence detector with wavelengths of excitation at 340 nm and emission at 450 nm. A gradient elution program was optimized for glutamate measurement with a flow rate 0.75ml/min. This program began at 100% mobile phase A, containing 40 mM sodium dihydrogen phosphate (the solution was adjusted to pH 7.8 with 10 M sodium hydroxide). At 17 min, 100% mobile phase B was used containing 45% acetonitrile and 45% methanol. Total analysis time was 30 min.

Isolation of extracellular vesicles

Extracellular vesicles were prepared as previously described (Skog *et al.* 2008) with total exosome isolation (from cell culture media) kit (Invitrogen). Briefly, human monocytes were cultured and differentiated for 7 d to become macrophages. Macrophages were treated with PM_{2.5} in vesicle-free medium (Neurobasal medium), and conditioned medium from 1.1×10^6 cells/well was collected after 24 h. Extracellular vesicles were purified by differential centrifugation. Macrophage-conditioned medium was centrifuged for 5 min at 300 g to remove cells. Supernatants were further centrifuged for 20 min at 4,000 g. Additional supernatants were mixed with half volume of total exosome isolation reagent and centrifuged at 10,000 g for 1 h. The extracellular vesicle pellets were resuspended in M-PER Protein Extraction Buffer (Pierce) for western blot or in neurobasal medium for HPLC after 48 h incubation with and without glutamine at 37°C.

Statistical analysis

Data were analyzed as means $\pm$ SEM. The data were evaluated statistically by the ANOVA, followed by the Tukey test for paired observations. Significance was considered with a p value < 0.05 .

Results

PM_{2.5} reduces the levels of tight junction proteins

Little is known whether PM_{2.5} could directly interact with brain cells. Although PM_{2.5} is able to infiltrate into circulation, it faces a significant obstacle to affect brain cells due to the structure of BBB. A key feature of BBB is the TJs formed by the monolayer of endothelial cells (ECs) lining the blood vessels in the brains (Daneman & Prat 2015). To determine the effect of PM_{2.5} on TJs, we used both monolayer cultures of HUVEC and HBMEC and treated them with doses of PM_{2.5} ranging from 6.25 to 100 $\mu\text{g/ml}$. PM_{2.5} exposure resulted in dose-dependent decreases in ZO-1 and ZO-2 protein levels in HUVEC (Fig. 1A, C and E) and HBMEC (Fig. 1B, D and F), as determined by western blot. Interestingly, TJs in HBMEC were more sensitive to PM_{2.5} toxicity compared with HUVEC; even a dose of 6.25 $\mu\text{g/ml}$ induced significant decreases in ZO-1 and ZO-2 protein levels in HBMEC. In comparison, PM_{2.5} did not significantly reduce ZO-1 and ZO-2 until relatively higher doses of 50 and 100 $\mu\text{g/ml}$ in HUVEC. Taken together, these results suggest that PM_{2.5} may have a selective toxicity toward the TJs of brain endothelial cells.

PM_{2.5} increases permeability and monocytes transmigration across the endothelial monolayer

To determine the effects of PM_{2.5} on the functional integrity of the endothelial monolayer, HUVEC were used to construct an artificial BBB based on a previous report (Persidsky *et al.* 1997). In the trans-well culture system, an endothelial monolayer was allowed to form in the upper chambers (inserts), while the lower chamber remained cell-free. We first added FITC Dextran-4, which is typically impermeable to intact endothelial monolayer, in the upper chambers and quantified the FITC Dextran-4 in lower chambers through fluorescent measurement (Fig. 2A). FITC Dextran-4 measurement in the monolayer-free lower chamber was set as the positive control. FITC Dextran-4 that traversed the HUVEC monolayer was significantly increased in PM_{2.5}-treated (25 $\mu\text{g/ml}$) group compared with the mock-treated group, suggesting that PM_{2.5} increases the permeability of endothelial monolayer (Fig. 2B). Furthermore, we determined the monocyte transmigration through the HUVEC monolayer. HUVEC monolayers were exposed to 0, 6.25, 12.5, 25, 50 or 100 $\mu\text{g/ml}$ of PM_{2.5} for 24 h. Monocytes (5×10^5 cells/well) were added to the upper chambers and their transmigration through HUVEC was quantified through counting the migrated monocytes in lower chambers (Fig. 2C). At the presence of PM_{2.5}, the number of migrated monocytes, as determined by DNA DAPI staining, significantly increased in a dose-dependent manner, compared with mock-treated control (Fig. 2D–E). These data suggest that PM_{2.5} impaired the functional integrity of TJs of the endothelial monolayer.

PM_{2.5} induces direct neurotoxicity

To determine the effect of PM_{2.5} on neurons *in vitro*, we have measured the viability of primary human neurons and RCN by MTT after their exposure to PM_{2.5}. Exposure to PM_{2.5} significantly decreased cell viability of human neurons in a dose-dependent manner. In contrast, RCN appeared to be less sensitive to PM_{2.5} toxicity compared with human neurons in the viability assay (Fig. 3A–B). Consistent with the viability assay, caspase 3 cleavage was significantly increased in primary human neurons while that in RCN was slightly increased after PM_{2.5} exposure, indicating the activation of programmed cell death (Fig. 3C–D). The loss of viability is specific to neurons, because PM_{2.5} exposure significantly reduced the neuronal antigen MAP2 in RCN (Fig. 3E–F), compared with mock-treated control group. These data suggest that PM_{2.5} has a direct neurotoxic effect *in vitro*.

PM_{2.5} increases macrophage-mediated neurotoxicity

When TJs in brain endothelial cells are damaged following PM_{2.5} exposure, inflammatory monocytes are likely recruited from circulation and differentiate into macrophages. If these macrophages, along with resident microglia, are not quickly controlled, they can become pathogenic and contribute to neurotoxicity (Wynn *et al.* 2013). Therefore, we determined whether PM_{2.5} increases macrophage- and microglia-mediated neurotoxicity. Human macrophages were derived from primary human monocytes and more than 99% were positively marked with CD68 (Fig. 4A) *in vitro*, suggesting a highly enriched macrophages culture. Exposure to PM_{2.5} did not reduce the cell viability of macrophages (Fig. 4B). Mock-treated control macrophages and microglia appeared to retain their characteristic morphology, while PM_{2.5}-treated cells became rounded as the exposure dose increased (Fig. 4C). After macrophages and microglia were exposed to PM_{2.5} (0, 6.25, 12.5, 25, 50 or 100 µg/ml) for 24 h, supernatants were collected and served as macrophage-conditioned media (MCM) and microglia-conditioned media (MGCM). Both MCM and MGCM were applied to RCN cultures accounting for 30% of total volume of the neuronal cultures for 24 h to test their neurotoxic potential. To visualize the extents of neuronal injury, we labeled the neuronal cultures with MAP2 and observed a significant decrease of MAP2 immunofluorescence, compared with the mock-treated control conditioned media (Fig. 4D–E). Exposure to MCM or MGCM also increased caspase 3 cleavage in neurons. The cleavage ratio of caspase 3 reached 2-fold with 50 µg/ml PM_{2.5}-treated MCM and 10-fold with 25 µg/ml PM_{2.5}-treated MGCM (Fig. 4F–G). The PM_{2.5}-treated MCM or MGCM dramatically reduced the cell viability of RCN compared with the untreated control group (Fig. 4H–I).

Neurotoxicity of PM_{2.5}-treated macrophage is dependent on glutamate production

High concentrations of extracellular glutamate induce neuronal damage. To determine whether PM_{2.5} increases glutamate production in macrophage and microglia, we measured the extracellular glutamate concentration through HPLC in human macrophage and microglia culture supernatants after PM_{2.5} exposure for 24 h. As the exposure doses of PM_{2.5} increased, the concentration of extracellular glutamate continued to increase in macrophage (Fig. 5A) and microglia (Fig. 5B). The RCN viability was inversely correlated with the glutamate concentration (Fig. 5C–D), suggesting a strong association of glutamate

to the neurotoxicity. To confirm the mechanism of PM_{2.5}-induced neurotoxicity, we pretreated RCN with MK801, an NMDA receptor antagonist, which effectively blocked NMDA receptor activation. Pretreatment with MK801 for 2 h blocked neurotoxicity mediated by PM_{2.5}-treated MCM (Fig. 5E–F), suggesting that PM_{2.5} causes neurotoxicity through excess glutamate production in macrophages.

PM_{2.5} induces glutaminase release in the extracellular vesicles

In macrophages, glutamate is mainly converted from glutamine by glutaminase. Therefore, we determined the glutaminase function, expression and localization in macrophages after PM_{2.5} exposure. We used BPTES, a previously described glutaminase inhibitor, to block the functional role of glutaminase in macrophages after PM_{2.5} exposure (Robinson *et al.* 2007). When BPTES was added to the macrophage culture along with PM_{2.5} for 24 h, BPTES significantly reduced the glutamate production in human macrophages (Fig. 6A), suggesting a functional role for glutaminase in excess glutamate generation in PM_{2.5}-treated macrophages. Furthermore, we targeted the regulation of glutaminase and found that both GAC and KGA showed a trend of decreasing following PM_{2.5} exposure (Fig. 6B–D), suggesting that PM_{2.5} may promote glutaminase release from the cells. To further test whether the glutaminase is released from macrophages following PM_{2.5} exposure, we tested the extracellular glutaminase activity through detection of glutamate production in supernatants, as well as the protein level of glutaminase. Glutamine-free supernatants were collected from both PM_{2.5}-treated and mock-treated control macrophages and divided into two parts. One part was supplied with 10 mM glutamine and the other part with glutamine-free medium. After 48 h incubation at 37°C, both were subjected to HPLC to determine the glutamate levels. Indeed, there was glutamate production in extracellular and the glutamate production significantly increased with the treatment of PM_{2.5} (Fig. 6E), suggesting that the released glutaminase increased its enzyme product glutamate. Since we have recently discovered that glutaminase is released from HIV-1-infected and immune activated macrophages through extracellular vesicles (Wu et al, manuscript in preparation), we further determined whether PM_{2.5} also could increase glutaminase release through extracellular vesicles. We subjected the MCM to extracellular vesicle isolation and found increased GAC protein levels in extracellular vesicles in PM_{2.5}-treated macrophages compared with mock-treated control macrophages (Fig. 6F). Collectively, these data suggest that PM_{2.5} may increase GAC release through extracellular vesicles and contribute to excess glutamate generation from macrophages leading to neurotoxicity.

Discussion

Exposure to higher levels of ambient airborne PM_{2.5} has been epidemiologically associated with sudden deaths, respiratory and cardiovascular illnesses (MohanKumar *et al.* 2008, Deng *et al.* 2013b, Deng *et al.* 2013a). In addition to effects on the respiratory system, the particles may elicit a systemic inflammatory response (Schwarze *et al.* 2010). In the current study, we evaluate the effect of PM_{2.5} on the CNS through our well-defined *in vitro* primary neuron, macrophage and microglia cultures. We found that PM_{2.5} induces damage and increases permeability in TJs of endothelial monolayer *in vitro*, resulting in increased monocytes transmigration across endothelial monolayer. Furthermore, PM_{2.5} treatment

decreased cell viability and neuronal antigen MAP2, suggesting that PM_{2.5} induces direct neurotoxicity. Interestingly, PM_{2.5} also induces indirect neurotoxicity by targeting macrophages. PM_{2.5} increased macrophage-mediated neurotoxicity when it was added to macrophage cultures. To further investigate the factors involved in the neurotoxicity, we determined the glutamate levels in the culture supernatants. PM_{2.5} exposure increased glutamate production in both macrophages and microglia. Pre-treatment with MK801 alleviated neuron loss, suggesting that PM_{2.5} causes neurotoxicity through excess glutamate production in macrophages. Furthermore, we showed increased extracellular glutamate production and GAC protein levels after PM_{2.5} exposure, suggesting that glutaminase release is essential for increased extracellular glutamate level. Collectively, we characterized adverse effects of PM_{2.5} on CNS *in vitro* and elucidate the mechanism of PM_{2.5}-induced neurotoxicity through vesicle-mediated glutaminase release and glutamate generation.

The PM_{2.5} concentrations used in the current study are environmentally relevant. According to a repeated-measure study, the mean person PM_{2.5} in office workers and truck drivers are 94.6 µg/m³ and 126.8 µg/m³, respectively (Baccarelli *et al.* 2014). Sun *et al.* suggested that 24 µg/ml residual oil fly ash in lung epithelial lining fluid is a reasonable approximation of possible human exposure (Sun *et al.* 2001). Accordingly, a person breathing 20 m³ of air containing a PM_{2.5} concentration of 150 µg/m³ for 24 h and depositing 20% of the inhaled PM in a volume of 4000 ml blood would have a PM_{2.5} concentration in the blood of 0.15 µg/ml. The deposition of PM accumulated with the exposure time (Oberdörster *et al.* 2002), makes it possible for the local PM_{2.5} to reach the concentrations used in our study.

The BBB is composed of brain microvascular endothelial cells (BMVEC), pericytes and astrocytes. Tight junctions interconnect the endothelial cells to ensure the structural integrity of the BMVEC monolayer and form a physical barrier restricting paracellular flux. Tight control of vascular permeability is essential for the homeostasis and functionality of the CNS (Garberg *et al.* 2005, Haorah *et al.* 2007). We report for the first time that direct PM_{2.5} treatment decreased tight junction protein expression levels and increased vascular permeability. This finding is consistent with the reports of nanoparticles on neurotoxicity (Chen *et al.* 2008, Hanada *et al.* 2014). Previous research demonstrated that tobacco smoke exposure increased the total generation of microvesicles from monocytes and macrophages (Li *et al.* 2010). Importantly, monocyte-derived microvesicles were found to disrupt endothelial cells integrity and rapidly induce endothelial apoptosis (Aharon *et al.* 2008). Therefore, it is possible that circulating PM_{2.5} has a direct effect on monocytes, impairing endothelial permeability prior to migration through the endothelium. Since PM_{2.5} is prevalent in the ambient atmosphere in many parts of the world, tight-regulation of PM_{2.5} is of critical importance to neurologic diseases.

Exposure to air pollutants causes neuroinflammation by elevated levels of pro-inflammatory factors such as interleukin-6 (IL-6), TNF-α and IL-1β (Levesque *et al.* 2011) and an altered brain innate immune response (Calderon-Garciduenas *et al.* 2008). In our study, we showed that PM_{2.5} exposure induced both direct and macrophage-mediated neurotoxicity. Beside the pro-inflammation effect determined by increased TNF-α level (data not shown), PM_{2.5} increased glutamate production. Glutamate is an excitatory neurotransmitter and the extracellular concentrations of glutamate should be rapidly removed to prevent it from

inducing neuronal injury (Ye *et al.* 2013, Huang *et al.* 2011). Indeed, patients with Parkinson's disease, Alzheimer's disease or other neuronal diseases showed elevated glutamate levels produced by microglia or macrophages (Huang *et al.* 2011, Erdmann *et al.* 2009). We report for the first time that PM_{2.5} exposure increases glutamate production by both macrophages and microglia, which is consistent with the detrimental role of these cell types during aberrant immune activation in the CNS.

Glutaminase is the primary enzyme responsible for the generation of glutamate by catalyzing the deamination of glutamine in the CNS (Zhao *et al.* 2004, Huang *et al.* 2011). BPTES, a glutaminase inhibitor, inhibited glutamate production in macrophages. In our previous study, excess levels of glutamate in HIV-1-infected microglia were due to elevated protein expression of glutaminase (Huang *et al.* 2011). In the current study, we determined elevated extracellular glutamate production and increased extracellular GAC protein level after PM_{2.5} exposure, which was consistent with recent studies (Tian *et al.* 2012, Ye *et al.* 2013). Extracellular vesicles are 30–100 nm in diameter and are shed from many different cell types under both normal and pathological conditions. Macrophages in the presence of tobacco smoke or liposomal antigens alter morphology and extracellular vesicles production (Li *et al.* 2010, Fortin *et al.* 2001). This is consistent with our observation for macrophages and microglia after PM_{2.5} exposure. The release of GAC is likely through extracellular vesicles because after PM_{2.5} exposure, the intracellular protein levels of GAC trended downward and increased extracellular levels of GAC were detected in extracellular vesicles. However, we were unable to detect a similar increase of KGA in the extracellular vesicles after PM_{2.5} exposure. This could be due to the low levels of KGA in extracellular vesicles that were below the detection limit of our assay. Whether both KGA and GAC subject to similar release through extracellular vesicles after PM_{2.5} exposure merits further investigation. The elevated GAC level in extracellular vesicles after PM_{2.5} exposure is of particular concern to the CNS because the extracellular GAC is capable of rapidly converting the abundant extracellular amino acid glutamine into excitotoxic levels of glutamate.

Conclusions

In summary, our study demonstrates that PM_{2.5} damages the blood-brain barrier *in vitro* and induces neurotoxicity directly or through a macrophage-mediated excitotoxicity. PM_{2.5} also induces release of macrophage glutaminase through extracellular vesicles, which underlies the detrimental effects of macrophage neurotoxicity following PM_{2.5} exposure.

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Abbreviations

BBB	blood-brain barrier
CNS	central nerves system
GAC	glutaminase C
HBMEC	human brain microvascular endothelial cells
HUVEC	human umbilical vein endothelial cells
KGA	kidney-type glutaminase
MCM	macrophage-conditioned media
MGCM	microglia-conditioned media
NMDA	N-Methyl-D-aspartate
PM	particulate matter
PM_{2.5}	particulate matter with an aerodynamic diameter 2.5 µm
RCN	rat cortical neuron
TJs	tight junctions
ZO-1	zonula occludens protein-1
ZO-2	zonula occludens protein-2

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Fig. 1. PM_{2.5} reduces the levels of TJ proteins in endothelial monolayer

A–B: The levels of TJ proteins ZO-1 and ZO-2 in HUVEC (A) and HBMEC (B) in both PM_{2.5}-treated groups and mock-treated control group were determined by western blot. β-actin was used as the loading control. C–F: Protein levels of ZO-1 and ZO-2 were normalized as a ratio to β-actin after densitometric quantification and presented as fold change relative to control. Results are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with the untreated control group.

Fig. 2. PM_{2.5} increases permeability and monocytes transmigration cross endothelial monolayer
 A: Scheme of BBB permeability. B: HUVEC monolayer permeability after treatment with PM_{2.5} (25 µg/ml) for 24 h. The monolayer-free upper chamber group was set as the positive control (PC). C: Scheme of monocyte migration. Cell types and their placement in the transwell system were indicated. D: Monocytes migrated through PM_{2.5}-treated HUVEC monolayers in the transwells were determined by immunostaining with DAPI. E: Quantification of migrated monocytes was performed by counting the number of DAPI-positive nuclei in each microscope field (n = 10). Scale bar, 50 µm. **p* < 0.05, ****p* < 0.001 compared with the untreated control group.

Fig. 3. PM_{2.5} induces direct neurotoxicity

A–B: Cell viability of human neurons (A) and RCN (B) after treatment with PM_{2.5} for 24 h. C–D: Caspase3 cleavage in human neurons (C) and RCN (D) after treatment with PM_{2.5} for 24 h were determined by western blot. β-actin was used as loading control. E: Representative MAP2 staining of primary RCN after treatment with PM_{2.5} or staurosporine for 24 h. Staurosporine (STS, 1 μM) was used as a positive control. F: Quantification of MAP2 fluorescence in panel E. Scale bar, 50 μm. Results are representative of three independent experiments. **p* < 0.05, ***p* < 0.01 compared with the untreated control group.

Fig. 4. PM_{2.5} increases macrophage-mediated neurotoxicity

A: Enrichment of macrophage cultures was evaluated by CD68 immunostaining. B: Cell viability of macrophages after PM_{2.5} exposure for 24 h. C: Morphology of macrophages (CD68-staining pictures) and microglia (bright-field pictures) after PM_{2.5} exposure. Typical rounded macrophages and microglia after PM_{2.5} exposure were marked with red arrows. D: Representative MAP2 staining of RCN after treatment with MCM. E: Quantification of MAP2 fluorescence in panel D. Scale bar, 50 μ m. F–G: Caspase 3 cleavage in RCN after treatment with MCM (F) or MGCM (G) was determined by western blot. β -actin was used as loading control. H–I: Cell viability of RCN after treatment with MCM (H) or MGCM (I). Results are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control MCM-treated group.

Fig. 5. Neurotoxicity of PM_{2.5}-treated macrophages is dependent on glutamate production
 A–B: HPLC determination of glutamate production in macrophages (A) and microglia (B) supernatants after PM_{2.5} treatment. C–D: Correlation of neuronal viability with extracellular glutamate levels. E: Representative MAP2 staining of RCN after treatment with MCM. RCN were pre-treated with 3 μM MK801 (NMDA receptor antagonist) for 2 h before treatment with MCM. Scale bar, 50 μm. F: Quantification of MAP2 fluorescence in panel E. Results are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control MCM-treated group.

Fig. 6. PM_{2.5} induces glutaminase release in the extracellular vesicles

A: HPLC determination of glutamate production in macrophage supernatants. Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES, 1 μM) was added to the culture along with PM_{2.5}. B: Protein levels of GAC and KGA in macrophages after exposure to PM_{2.5} were determined through western blot. C-D: Quantification of GAC and KGA in panel B. E: Extracellular glutaminase activity. Glutamate levels in supernatants were determined through HPLC after incubation with (black) or without (white) glutamine for 48 h at 37°C. F: Extracellular glutaminase level. KGA and GAC protein levels in both whole cell lysates and extracellular vesicles were determined by western blot. Results are representative of three independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 compared with the untreated control group.