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Nanometer size diesel exhaust particles are selectively toxic to dopaminergic neurons: the role of microglia, phagocytosis, and NADPH oxidase

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ABSTRACT

The contributing role of environmental factors to the development of Parkinson's disease has become increasingly evident. We report that mesencephalic neuron-glia cultures treated with diesel exhaust particles (DEP; 0.22 μM) (5-50 μg/ml) resulted in a dose-dependent decrease in dopaminergic (DA) neurons, as determined by DA-uptake assay and tyrosine-hydroxylase immunocytochemistry (ICC). The selective toxicity of DEP for DA neurons was demonstrated by the lack of DEP effect on both GABA uptake and Neu-N immunoreactive cell number. The critical role of microglia was demonstrated by the failure of neuron-enriched cultures to exhibit DEP-induced DA neurotoxicity, where DEP-induced DA neuron death was reinstated with the addition of microglia to neuron-enriched cultures. OX-42 ICC staining of DEP treated neuron-glia cultures revealed changes in microglia morphology indicative of activation. Intracellular reactive oxygen species and superoxide were produced from enriched-microglia cultures in response to DEP. Neuron-glia cultures from NADPH oxidase deficient ($PHOX^{-/-}$) mice were insensitive to DEP neurotoxicity when compared with control mice ($PHOX^{+/+}$). Cytochalasin D inhibited DEP-induced superoxide production in enriched-microglia cultures, implying that DEP must be phagocytized by microglia to produce superoxide. Together, these in vitro data indicate that DEP selectively damages DA neurons through the phagocytic activation of microglial NADPH oxidase and consequent oxidative insult.

Key words: dopaminergic neurotoxicity • oxidative stress • Parkinson's disease

Parkinson's disease (PD) is a devastating illness of slow and progressive dopaminergic (DA) neurodegeneration where the majority of reported cases result from unknown etiology. There is increasing support that several environmental toxins induce inflammation and may trigger neurodegenerative disease, where microglia are implicated as the pivotal cell type mediating neuroinflammatory damage (1–5). The following study sought to determine whether components of air pollution, such as diesel exhaust particles (DEP), might

also be selectively toxic to DA neurons through microglial activation.

Particulate matter (PM) is a ubiquitous component of urban air pollution that is epidemiologically associated with increased morbidity and mortality in respiratory and cardiovascular disease (6). DEP are a category of PM derived from diesel fossil fuels and combustible engines (6). PM is divided into three major size categories: ultra-fine (<0.1 μM), fine (<2.5 μM), and coarse (<10 μM and >2.5 μM). Ultra-fine particles are more likely to enter circulation and are associated with the major oxidative and proinflammatory effects of PM (7–10). DEP particles (<0.22 μM) were used in this study to investigate whether DEP can trigger inflammation-mediated DA neurodegeneration in vitro.

There have been increasing reports that PM can enter the brain and that PM may be associated with neurodegenerative pathology in vivo. Inhaled PM is systemically distributed and can be found in several organs including bone marrow, liver, and brain (11). Interestingly, when the brains from highly PM-exposed Mexico City dogs were compared with control dog brains from less polluted cities, several neuropathological markers were found, including expression of nuclear neuronal NF-κB and iNOS in cortical endothelial cells (12). Further, physical damage to the blood-brain barrier (BBB) was reported in the highly PM-exposed Mexico City dogs, along with neurodegenerative pathology (12). Degenerating cortical neurons, apoptotic glial white matter cells, non-neuritic plaques, and neurofibrillary tangles were also found in dogs with high PM exposure (12), thus supporting an association between ambient PM and neuropathology. In a separate study, dogs chronically exposed to air pollutants showed an increase in glial cell number, iNOS expression, COX2 expression, neuronal β amyloid (1–42) expression, and higher levels of diffuse plaques, suggesting that exposure of air pollutants is associated with evidence of chronic brain inflammation and neurodegeneration (13). In another experiment, rats chronically exposed to PM were shown to have a higher content of brain glial fibrilary acid protein (14), which is indicative of gliosis. Further, the extreme DEP exposure that occurs with underground mining has also been associated with organic brain damage (15). Although these data are suggestive of DEP and PM associated neuropathology, the mechanistic details of PM neurotoxicity are lacking.

Previous work from our laboratory has used the neuron-glia culture system as an efficient in vitro screen for both inflammation-mediated neurotoxic and neuroprotective compounds affecting DA neuron survival, where in vitro results effectively predicted the outcome from continuing experiments using in vivo systems (16, 17). Thus, the purpose of the following study was to use this neuron-glia model to investigate the hypothesis that DEP can activate microglia and induce oxidative stress, which subsequently results in DA neurotoxicity.

METHODS

Animals

Timed-pregnant (gestational day 14) adult female Fisher 344 rats were purchased from Charles River Laboratories (Raleigh, NC). Eight-week-old (25-30g) male and female B6.129S6-*Cybb*^{tm1Din} (PHOX$^{-/-}$) and C57BL/6J (PHOX$^{+/+}$) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in a strict pathogen free environment. The PHOX$^{-/-}$ mice lack the functional catalytic subunit of the NADPH oxidase complex gp91. NADPH oxidase is an

inducible electron transport system in phagocytic cells that is responsible for the generation of the respiratory burst. $PHOX^{-/-}$ mice are unable to generate extracellular superoxide in response to LPS or other immunological stimulus. Breeding of the mice was designed to achieve accurate timed-pregnancy ± 0.5 days. The $PHOX^{-/-}$ mutation is maintained in the C57BL/6J background; thus the C57BL/6J ($PHOX^{+/+}$) mice were used as control animals. Housing, breeding, and experimental use of the animals were performed in strict accordance with the National Institutes of Heath guidelines.

Reagents

DEP (2975 Industrial Forklift) were purchased from the National Institute of Standards and Technology (Gaithersburg, MD). Lipopolysaccharide (strain O111:B4) was purchased from Calbiochem (San Diego, CA). Cell culture ingredients were obtained from Life Technologies (Grand Island, NY). [^{3}H]dopamine (DA, 28 Ci/mmol) and [2,3 -^{3}H]GABA (81 Ci/mmol) were purchased from NEN Life Science (Boston, MA). The polyclonal antibody against tyrosine hydroxylase (TH) was a kind gift from Dr. John Reinhard of GlaxoSmithKline (Research Triangle Park, NC). The neuron-specific nuclear protein (Neu-N) monoclonal antibody and the monoclonal antibody raised against the CR3 compliment receptor (OX42) were obtained from PharMingen (San Diego, CA). The Biotinylated horse anti-mouse and goat anti-rabbit secondary antibodies were purchased from Vector Laboratories (Burlingame, CA). Catalase was obtained from Calbiochem (San Diego, CA). 2',7'-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Calbiochem (La Jalla, CA). WST-1 was purchased from Dojindo Laboratories (Gaithersburg, MD). Tumor necrosis factor α (TNF-α) ELISA kits were purchased from R&D Systems Inc. (Minneapolis, MN). Prostaglandin E_2 (PGE_2) ELISA kits were purchased from Cayman Chemical Company (Ann Arbor, MI). All other reagents came from Sigma Aldrich Chemical Co. (St. Louis, MO).

Preparation of nanometer-sized DEP

DEP were desiccated at room temperature until use. DEP were prepared by vortexing (<20 s) a 1 mg/ml concentration of DEP in treatment media, followed by sonication for 20 min. A sterile Millipore (Bedford, MA) 0.22 μM syringe filter was first washed with sterile treatment media and then used to filter the sonicated DEP solution. The DEP treatment media were then immediately diluted to the appropriate concentrations and exposed to the culture.

Mesencephalic neuron-glia cultures

Rat and mouse ventral mesencephalic neuron-glia cultures were prepared using a described previously protocol (18). Briefly, midbrain tissues were dissected from day 14 Fisher 344 rat embryos or day 14 mouse embryos ($PHOX^{+/+}$ or $PHOX^{-/-}$). Cells were dissociated via gentle mechanical trituration in minimum essential medium (MEM) and immediately seeded ($5x10^5$/well) in poly-D-lysine (20 μg/ml) precoated 24-well plates. Cells were seeded in maintenance media and exposed to the treatment media described previously (18). Three days after seeding, the cells were replenished with 500 μl of fresh maintenance media. Cultures were exposed 7 days after seeding.

Microglia-enriched cultures

Primary enriched microglia cultures were prepared from the whole brains of day-old Fisher 344 rat pups, using the procedure described previously (19). Briefly, the menengies and blood vessels were removed, and the brain tissue was gently triturated and seeded ($5x10^7$) in 150 cm^3 flasks. One week after seeding, the media were replaced. Two weeks after seeding, when the cells had reached a confluent monolayer of glial cells, microglia were shaken off and either replated at 1×10^5 in a 96-well plate or reseeded on top of a neuron-enriched culture. Cells were treated 24 h after seeding the enriched microglia.

Neuron-enriched cultures

Mesencephalic neuron-glia cultures were seeded (5×10^5/well) in 24-well plates precoated with poly-D-lysine. Twenty-four hours postseeding, 5-10 μM cytosine β-D-arabinofuranoside was added to the culture. After 2 days, the cytosine β-D-arabinofuranoside was removed and replaced with fresh media. Neuron-enriched cultures are 98% pure, as indicated by ICC staining with OX-42 and GFAP antibodies. Neuron-enriched cultures were treated for 8-9 days postseeding.

For microglia add-back cultures, the microglia was plated on top of the neuron-enriched culture at 6 days postseeding, resulting in the addition of either 10% (500 μl of 1×10^5) or 20% (500 μl of 2×10^5) microglia. Cells were treated 7 days after the initial seeding of the neuron-enriched cultures.

Uptake assays (DA and GABA)

Cells were incubated in Krebs-Ringer buffer (16 mM NaH_2PO_4, 16 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM EDTA, 4.7 nM KCL, 16 mM Na_2HPO_4) for 15 min at 37°C with either 5 μM [^{3}H]GABA or 1 μM [^{3}H]DA. Nonspecific uptake was blocked for GABA with 10 μM NO-711 and 1 mM β alanine. Nonspecific uptake was blocked for DA with 10 μM mazindole. After incubation, cells were washed three times with 1 ml/well of ice-cold Krebs-Ringer buffer. Cells were then lysed with 0.5 ml/well of 1 N NaOH and mixed with 15 ml of scintillation fluid. Radioactivity was measured on a scintillation counter, where specific [^{3}H]GABA or [^{3}H]DA uptake was calculated by subtracting the mazindole or the NO-711 and β-alanine counts from the wells without the uptake inhibitors.

Immunostaining

Neurons were stained with the antibody against Neu-N, a neuronal nuclear protein used to identify neuron cell bodies. Microglia were stained with the monoclonal antibody raised against the CR3 receptor OX-42. Astrocytes were stained with the antibody against glial fibrillary acidic protein (GFAP), an intermediate filament protein that is synthesized only in astrocytes. Dopamine neurons were detected with the polyclonal antibody against tyrosine hydroxylase (TH). Briefly, cells were fixed for 20 min at room temperature in 3.7% formaldehyde diluted in phosphate buffered saline (PBS). After being washed twice with PBS, the cultures were treated with 1% hydrogen peroxide for 10 min. The cultures were again washed three times with PBS and then incubated for 40 min with blocking solution [PBS containing 1% bovine serum albumin (BSA), 0.4% Triton X-100, and 4% appropriate serum: normal horse serum for Neu-N, or OX-42

and normal goat serum for TH or GFAP staining]. The cultures were incubated overnight at 4°C with the primary antibody diluted in DAKO antibody diluent, and the cells were washed three times for 10 min each in PBS. The cultures were next incubated for 1 h with PBS containing 0.3% Triton X-100 and the appropriate biotinylated secondary antibody (Neu-N or OX-42: horse anti-mouse antibody, 1:227; TH or GFAP: goat anti-rabbit antibody). After being washed three times with PBS, the cultures were incubated for 1 h with the Vectastain ABC reagents diluted in PBS containing 0.3% Triton X-100. Cells were then washed two times with PBS, and the bound complex was visualized by incubating cultures with 3,3′-diaminobenzidine. Color development was halted by removing the reagents and washing the cultures twice with fresh PBS. To quantify cell numbers, nine representative areas per well in the 24-well plate were counted under the microscope at x100 magnification by two individuals in a blind experimental design. The average of these scores was reported.

Superoxide assay

Extracellular superoxide (O_2^-) production from microglia was determined as reported previously (20) by measuring the superoxide dismutase (SOD) inhibitable reduction of 2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2,4,-disulfophenyl)-2H-tetrazolium, monosodium salt, WST-1 (16, 21, 22). The assay was performed with Hanks' balanced salt solution (HBSS) without serum or other additives, as previously reported (1, 23). Briefly, 200 μl of primary enriched-microglia were seeded (1×10^5/well) in 96-well plates. The cells were then incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2-95% air. Immediately before treatment, cells were washed twice. To each well, 100 μl of HBSS with or without SOD (600 U/ml), 50 μl of vehicle or LPS, and 50 μl of WST-1 (1 mM) in HBSS were added. The cultures were incubated for 30 min at 37°C and 5% CO_2-95% air. The absorbance at 450 nm was read with a Spectra Max Plus microtiter plate spectrophotometer (Molecular Devices, Sunnyvale, CA). Cell free experiments with and without DEP were conducted to determine that DEP did not alter absorbance by itself. The amount of SOD-inhibitable superoxide was calculated and expressed as a percentage of vehicle-treated control cultures.

Intracellular reactive oxygen species assay

The production of intracellular reactive oxygen species (ROS) was measured by DCFH oxidation. The DCFH-DA reagent passively enters cell where it is de-acetylated by esterase to nonfluorescent DCFH. Inside the cell, DCFH reacts with ROS to form DCF, the fluorescent product. For this assay, 10 mM DCFH-DA was dissolved in methanol and was diluted 500-fold in HBSS without serum or other additives, to give a 20 μM concentration of DCFH-DA, as previously reported (1, 23). Enriched-microglia cultures seeded (5×10^4) in 96-well plates were then exposed to DCFH-DA for 1 h, followed by treatment with HBSS containing several concentrations of LPS or DEP for 2 h. After incubation, the fluorescence was read at the 485 nm excitation and 530 nm emission on a fluorescence plate reader. Cell free experiments with and without DEP were conducted to determine that DEP did not alter florescence by itself. To calculate the amount of intracellular ROS produced, the mean control treatment was subtracted from the mean treatment group.

TNF-α assay and PGE_2 assay

The production and release of TNF-α into the media was measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems (Minneapolis, MN), as described previously (18). The PGE_2 release was measured with a commercial ELISA kit from Cayman Chemical Company (Ann Arbor, MI).

Nitrite assay

As an indicator of nitric oxide production, the amount of nitrite accumulated in culture supernatant was determined with a colorimetric assay using Griess reagent [1% sulfanilamide, 2.5% H_3PO_4, 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride] (24), as previously reported (25). Briefly, 50 µl of Griess reagent and 50 µl of culture supernatant were incubated in the dark at room temperature for 10 min. After incubation, the absorbance at 540 nm was determined with the Spectra Max Plus microplate spectrophotometer. The sample nitrite concentration was determined from a sodium nitrite standard curve.

Statistical analysis

The data are expressed as either the percentage of control or the difference from control, where control values were set to either 100% or 0 accordingly. The treatment groups are expressed as means ± SE, and statistical significance was assessed with an ANOVA (ANOVA) followed by Bonferroni's multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

RESULTS

DEP are toxic to DA neurons

The neurotoxic effect of DEP on dopaminergic neurons was compared in rat mesencephalic neuron/glia cultures. Exposure to high concentrations of DEP (200 µg/ml and above) resulted in general toxicity to both neurons and microglia (data not shown). Concentrations of DEP failing to show toxicity to microglia (50 µg/ml and below) were used in this study. To discern the effect DEP on DA neuron function, the ability of cells to uptake [^{3}H]DA was measured. Addition of DEP to neuron-glia cultures resulted in a significant dose-dependent reduction in DA uptake ($P<0.05$; Fig. 1*A*), where the highest dose of 50 µg/ml DEP produced a 51% loss of DA neuron cell function. To quantitate the DA cell loss associated with DEP treatment, neuron/glia cultures were exposed to DEP and stained with the TH antibody, where the number of TH positive neurons was counted. LPS was used as a positive control for microglia-mediated neurotoxicity, where 5 ng/ml LPS resulted in a 66% reduction in TH-immunoreactive (TH-IR) cells. DEP reduced the number of TH-IR neurons in a dose-dependent manner ($P<0.05$; Fig. 1*B*), where the highest dose of 50 µg/ml DEP produced a 41% loss of DA neurons. These results are consistent with previous studies (20, 23, 26) where the inhibition of DA cell function is often the more sensitive measure, when compared with TH-IR cell loss. The increased sensitivity of DA uptake reflects the time lag between the two measures. Neurons die over time, and the cells that are living and damaged will have reduced function, while still staining with the anti-TH antibody.

DEP neurotoxicity is selective for DA neurons

Neuron-glia cultures exposed to DEP were compared for the ability to uptake [^{3}H]DA and [^{3}H]GABA. LPS was used as a positive control for microglia activation and selective DA neurotoxicity, where 5 ng/ml LPS resulted in a 77% reduction of DA uptake, while there was no significant difference in GABA uptake, compared with control (P<0.05). Figure 2*A* shows that only DA uptake was reduced by the addition of DEP to neuron-glia cultures, while GABA uptake remained unchanged (P<0.05). The number of Neu-N immunoreactive neurons in neuron-glia cultures was also counted to determine the toxic effect of DEP on the overall neuron number (Fig. 2*B*). LPS was used as a positive control for microglia activation and selective DA neurotoxicity, where LPS treatment resulted in no significant difference in the number of Neu-N positive cells, compared with control (P<0.05). These data also showed no significant effect of DEP on the number of Neu-N positive cells present in neuron-glia cultures (P<0.05). The lack of effect on Neu-N cell counts indicates that although DEP will decrease DA neuron number, the overall neuron number was not affected by DEP. Together, the lack of DEP effect on both GABA uptake and Neu-N cell count in neuron-glia cultures supports the DA selectivity of DEP-induced neurotoxicity.

Microglia mediate DEP-induced DA neurotoxicity

To investigate the role of microglia in DEP-induced DA neurotoxicity, [^{3}H]DA uptake was compared in neuron-enriched cultures vs. neuron-glia cultures. LPS was used as a positive control for microglia-mediated neurotoxicity, where 5 ng/ml LPS reduced DA uptake by 50% and there was no significant difference in GABA uptake compared with control (P<0.05). Although DEP remained toxic to DA neurons in the neuron-glia culture (P<0.05), the DA uptake in neuron-enriched cultures was not affected (Fig. 3*A*), demonstrating that DEP concentrations of 50 μg/ml and less were not directly toxic to DA neurons. Further, enriched-microglia (10 and 20%) were added back to neuron-enriched cultures and then treated with DEP to elucidate the role of microglia in DEP neurotoxicity. Figure 3*B* demonstrates that the addition of microglia to neuron-enriched cultures re-instated DEP-induced DA neurotoxicity (P<0.05), where the addition of larger numbers of microglia resulted in greater DA neurotoxicity.

DEP activate microglia

It has been well established that the activation of microglia and the consequent production of proinflammatory factors has been linked to DA neurotoxicity (23, 27). The supernatant from DEP treated neuron-glia cultures was tested for the presence of several proinflammatory factors. Analysis of supernatant collected at 3 h, 6 h, 12 h, 24 h, 4 days, 6 days, 8 days, and 9 days post-DEP treatment in neuron-glia cultures revealed that TNF-α, nitrite (indicative of nitric oxide production), and PGE_2 were not produced (data not shown). These results are consistent with previous studies where microglial activation resulted in the production of extracellular superoxide without the production of TNF-α and other proinflammatory factors in the case of rotenone (28), paraquat (26), and low concentrations of the Aβ peptide (20). However, at 12 h post-DEP treatment, ICC staining with the OX-42 antibody to the CR3 receptor present on microglia showed activated microglia morphology (Fig. 4). LPS was used as a positive control for microglia activation. The activated microglia are identified by the increase in staining intensity, the increase in the number of immunoreactive cells, and a characteristic, altered

ameboid morphology when compared with the control group. This early evidence of microglial activation occurred 8 days before the determination of DA neurotoxicity and supported the role of microglia as the triggering event of DEP-induced DA neurotoxicity.

DEP increases microglia ROS

There is increasing evidence that ROS are critical components of the proinflammatory signaling pathway in phagocytic cell (23). To further support the activation of microglia by DEP, intracellular ROS was measured in enriched-microglia cultures. LPS was used as a positive control for microglia activation. Figure 5*A* indicates a significant DEP dose-dependent increase in intracellular ROS in enriched-microglia cultures ($P<0.05$).

The production of extracellular ROS from activated microglia has also been strongly linked to DA neurotoxicity (2, 3, 20, 23, 28). Another characteristic of the activated phagocyte is the respiratory burst associated with the production of extracellular superoxide. The results presented in Fig. 5*B* demonstrate that the addition of DEP to enriched-microglial cultures induced a dose-dependent increase in extracellular superoxide, where 50 μg/ml DEP resulted in a 287% increase from control levels ($P<0.05$). LPS was used as a positive control for microglia activation, where addition of 100 ng/ml LPS resulted in a 233% increase from control levels.

In particular, DA neurons are more susceptible to oxidative stress, such as superoxide and hydrogen peroxide, due to reduced antioxidant capacity, as evidenced by low levels of intracellular glutathione in the DA neuron, when compared with other cell types (29). In general, toxins that induce oxidative stress will often demonstrate selective DA neurotoxicity at lower concentrations (20, 28) and graduate to general toxicity as the concentration increases. Consistent with this notion, here we show that DEP demonstrates general toxicity at higher doses (200 μg/ml and above), while remaining selectively toxic to DA neurons at lower concentrations (50 μg/ml and below). As our results also show that DEP induces the microglial production of superoxide, these data strongly suggest that the mechanism through which DEP is selectively toxic to DA neurons is through oxidative insult.

DEP-induced microglia mediated neurotoxicity is mediated by NADPH oxidase

To test the importance of extracellular superoxide for DEP-induced DA neurotoxicity, DEP-induced DA neurotoxicity in $PHOX^{-/-}$ mouse mesencephalic neuron-glia cultures was compared with $PHOX^{+/+}$ mouse cultures. $PHOX^{-/-}$ mice lack the functional gp91 protein, the catalytic subunit of the NADPH oxidase complex, and thus have no phagocytic respiratory burst. DA uptake at 8 days posttreatment was measured to determine the DEP neurotoxicity. Although DEP was toxic to DA neurons in the $PHOX^{+/+}$ culture ($P<0.05$), the DA uptake in $PHOX^{-/-}$ cultures was not affected by DEP (Fig. 6), demonstrating the pivotal role of NADPH oxidase generated ROS in DEP-induced DA neurotoxicity.

Phagocytosis mediates the microglial superoxide response to DEP

It has been suggested that some effects of DEP in lung tissue have been linked to the phagocytosis of the DEP particle (6, 30). Further observations from Veronesi's laboratory show that DEP is phagocytized by microglia (unpublished data). To determine if the phagocytosis of

DEP is required for the microglial production of superoxide, cytochalasin D was used to inhibit actin polymerization, thus immobilizing the microglial cytoskeleton and impairing phagocytosis. The addition of 50 μg/ml DEP produced a 400% increase in superoxide, compared with control. Figure 7 indicates that 15 min preincubation with cytochalasin D abolished the microglial superoxide response to DEP, supporting the role of phagocytosis in the DEP-induced activation of microglia. Cytochalasin D alone did not significantly alter superoxide production, compared with control values ($P<0.05$).

DISCUSSION

In the present study, we show that nanometer-sized DEP are selectively toxic to DA neurons through microglia-mediated ROS production. A predominant pathology of PD is the specific degeneration of DA neurons, while other neuronal subtypes remain generally unaffected. The neuron-glia culture model used in this study demonstrates that DEP neurotoxicity is selective to DA neurons (Fig. 2) and presents DEP as a potential candidate for the emerging class of environmental neurotoxins with microglia-mediated mechanisms (Fig. 3). Although Calderon-Garciduenas et al. (12) suggest that the persistent pulmonary inflammation and deteriorating olfactory and respiratory barriers associated with PM exposure may play a role in PM-induced neuropathology, the in vivo mechanisms are poorly defined and future studies will need to confirm the presence of selective DA neurotoxicity with chronic animal studies. Current research in our laboratory is focused on the characterization of the neurodegenerative consequences of DEP exposure in vivo.

Distribution studies have demonstrated that injected radiolabeled nanometer-sized iridium particles are found in the brain (31) and that inhaled ultra-fine silver particles are systemically distributed to various organs (heart, lung, kidney, spleen, and blood), including the brain (11). Such data support that PM can cross the blood brain barrier to reach the brain. Given the results from this study, this is of considerable concern, as some of the higher polluted cities, such as Los Angeles, report daily doses of PM as high as 300 μg/day (6). It is interesting to note that in the current study, a concentration as low as 5 μg/ml of DEP was shown to be toxic to DA neurons in vitro (Fig. 1*B*). Calderon-Garciduenas et al. (12) suggest that many neurodegenerative disorders may begin early in development and that air pollutants may be a key contributing factor. This raises further concern for the consequences of the cumulative effects of long-term DEP exposure and supports future epidemiological and experimental studies to determine the effects of PM and DEP exposure in concert with other environmental DA neurotoxins. Although the association of rural living and PD has been well documented (32), the results from this study suggest that urban living in cities associated with high PM concentrations could also be associated with increased risk of PD. Epidemiological studies assessing urban-living and work-related PM exposure, along with the associated increase in mortality linked to high particulate exposure, could perhaps unveil another susceptible population.

The mechanism of DEP DA selectivity is likely due to the generation of oxidative insult from microglia. DA neurons possess reduced antioxidant capacity, as evidenced by low intracellular glutathione that render DA neurons more vulnerable to oxidative stress and microglial activation, relative to other cell types (29). Additionally, the mesencephalon houses the SN and contains 4.5 times as many microglia when compared with the cortex (33). In the present study, our data show that DEP activate microglia (Fig. 4), that microglia are crucial to DEP-induced DA

neurotoxicity (Fig. 3), that DEP stimulated microglia to produce free radicals (superoxide; Fig. 5), and that NADPH oxidase is the source of DEP-induced microglial ROS responsible for DA neurotoxicity (Fig. 6*A*). Taken together, this suggests that DA neurons in the SN could be particularly vulnerable to DEP. However, higher concentrations of DEP may indeed be toxic to multiple cell types, including microglia.

The toxicity and immune-stimulating characteristics of DEP in the lung have been linked to both the adsorbed chemicals on the outside of the carbon particle and the physical characteristics of the particle itself (6). Although LPS is one of the common chemicals adsorbed on the surface of DEP (6), it is unlikely that this explains DEP neurotoxicity, as the cytokine proinflammatory response that is typical of LPS (TNF-α, NO, and PGE_2) was absent in this study. Several compounds known to be toxic to DA neurons (polyaromatic hydrocarbons, quinones, and transition metals) are also found on diesel particles (6). However, most of the potentially neurotoxic compounds adsorbed to DEP induce oxidative stress through redox-cycling with cytochrome p450 activity (6, 34) and not through NADPH oxidase activation, as suggested in the present study (Fig. 6*A*). Oxidative stress and inflammatory changes may however, be related to physicochemical features of PM. For example, work by Veronessi et al. (35, 36) report that the inflammatory response in respiratory epithelial cells to PM relates to physiochemical features of the particles, such as surface charge. Of further interest, carbon black particles, which lack all chemical and biological adsorbed compounds, have been shown to produce oxidative stress in cells through the NADPH oxidase pathway (6). Together, this supports that physiochemical factors of DEP are culpable in microglia activation.

Phagocytosis is one such process that is dependent on the physical characteristics of the stimulus. Other work has demonstrated that DEP is phagocytized by microglia (Veronesi, unpublished data). Although not all phagocytosis results in the respiratory burst (37), NADPH oxidase is present in phagocytic cells, such as microglia, and is often activated during phagocytosis to produce the respiratory burst. The activation of NADPH oxidase in the professional phagocyte during phagocytosis is dependent on the receptors or receptor complex identifying what is being internalized (38). In this study, we have demonstrated the importance of NADPH oxidase and the respiratory burst for DEP-induced DA neurotoxicity (Fig. 6*A*). However, we have also shown that inhibition of DEP phagocytosis will also inhibit the DA-neurotoxic respiratory burst from microglia (Fig. 6*B*). Future work in our laboratory is aimed at identifying the phagocytic receptors on microglia responsible for the DEP-induced activation of NADPH oxidase.

Recent work has shown that the microglial phagocytosis of dying neurons results in the death of purkinje cells during normal development, due to the production of ROS from the microglial respiratory burst (39). Our current work is one of the first studies to suggest the neurotoxic role of phagocytosis in microglial over-activation. Figure 7 depicts the proposed mechanism of phagocytosis-mediated DA neurotoxicity suggested by this research. Although phagocytosis is a common and necessary element to maintain homeostasis and remove cellular debris, this study suggests that the deleterious, oxidative collateral-damage of phagocytosis may be another characteristic of the over-activated microglia in the neurodegenerative disease state. This finding has broad-reaching implications, as several pathological hallmark proteins associated with neurodegenerative disease, such as myelin (40), melanin (41), prions (42), and β-amyloid (43, 44), are reported to be phagocytized by microglia. Of further concern, apoptotic and dying DA neurons are also reported to be phagocytized by microglia (45, 46), suggesting a potential

mechanism of ongoing and cyclic generation of oxidative insult in response to DA neurodegeneration. Oxidative stress has long been defined as one of the major contributing factors to many neurodegenerative diseases. Thus, understanding the role of phagocytosis in the neurodegenerative process may help to elucidate the difference between normal microglial homeostasis and the disease state, offering hope for the generation of novel therapeutic compounds.

In summary, this study reports the DA-selective neurotoxic characteristics of DEP in vitro, where toxicity was dependent on the presence of microglia and oxidative insult through the activation of NADPH oxidase. Further, we also report that the mobility of the microglial cytoskeleton is mandatory for the generation of DEP-induced superoxide. This strongly supports the role of phagocytosis as one of the contributing mechanisms to microglial-derived oxidative damage and suggests a new class of neurotoxic compounds, defined by the microglial phagocytic activation of the respiratory burst and the consequent collateral neuronal damage.

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Fig. 1

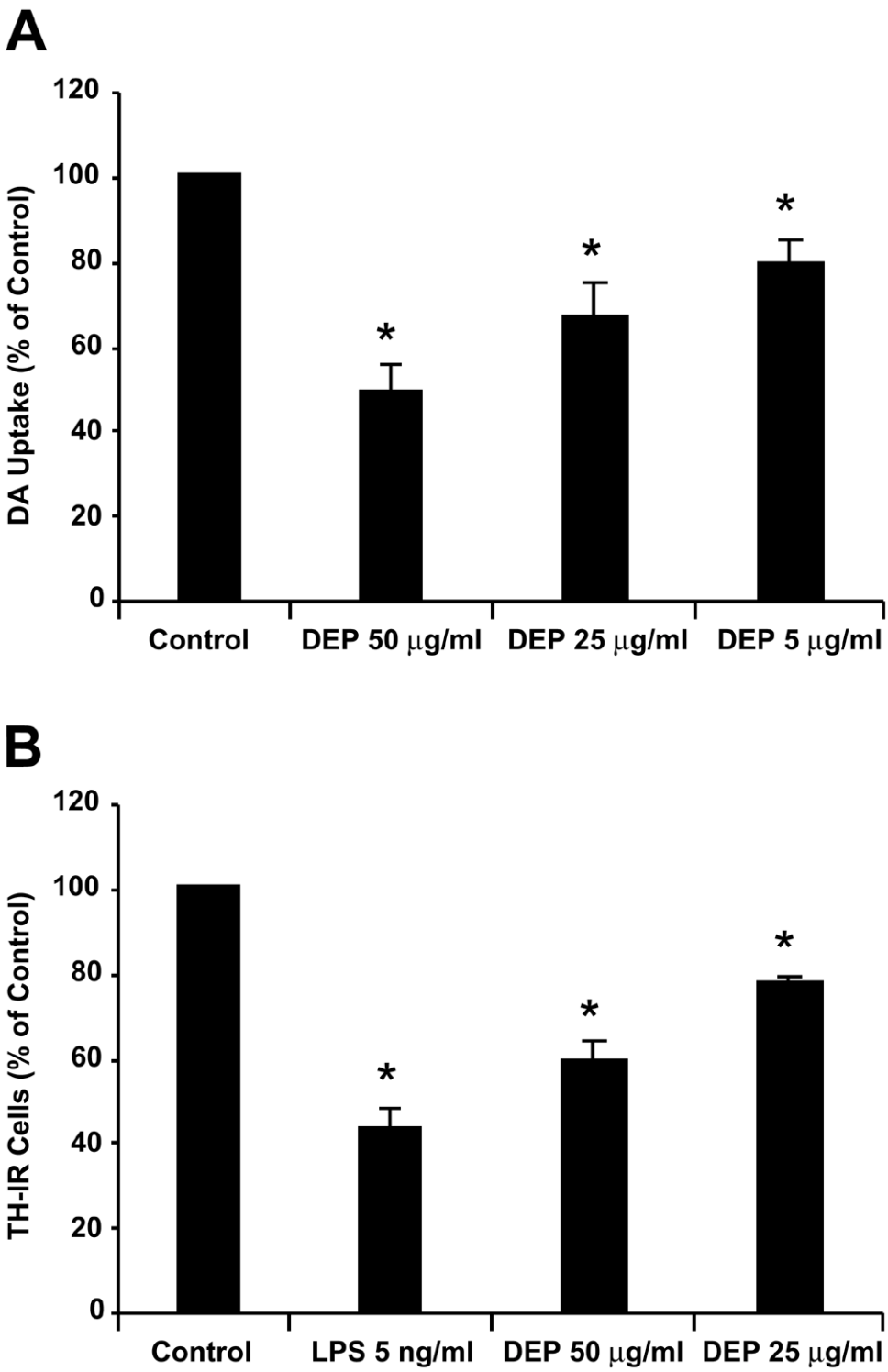

Figure 1. Diesel exhaust particles (DEP) are toxic to dopaminergic (DA) neurons. Mesencephalic midbrain neuron-glia cultures were treated with either vehicle, 5 ng/ml LPS, 50 μg/ml DEP, 25 μg/ml DEP, or 5 μg/ml DEP. LPS was used as a positive control for microglia-mediated DA neurotoxicity. ***A*)** DA neurotoxicity was measured at 8-9 days posttreatment using the [^{3}H]DA uptake assay. ***B*)** DA cell death was determined at 8-9 days posttreatment with immunocytochemical staining using the tyrosine hydroxylase antibody and counting the number of TH positive neurons present. Data are percentage of control cultures, are means ± SE, and are the average of 3 separate experiments. *Significant difference (P<0.05) compared with control.

Fig. 2

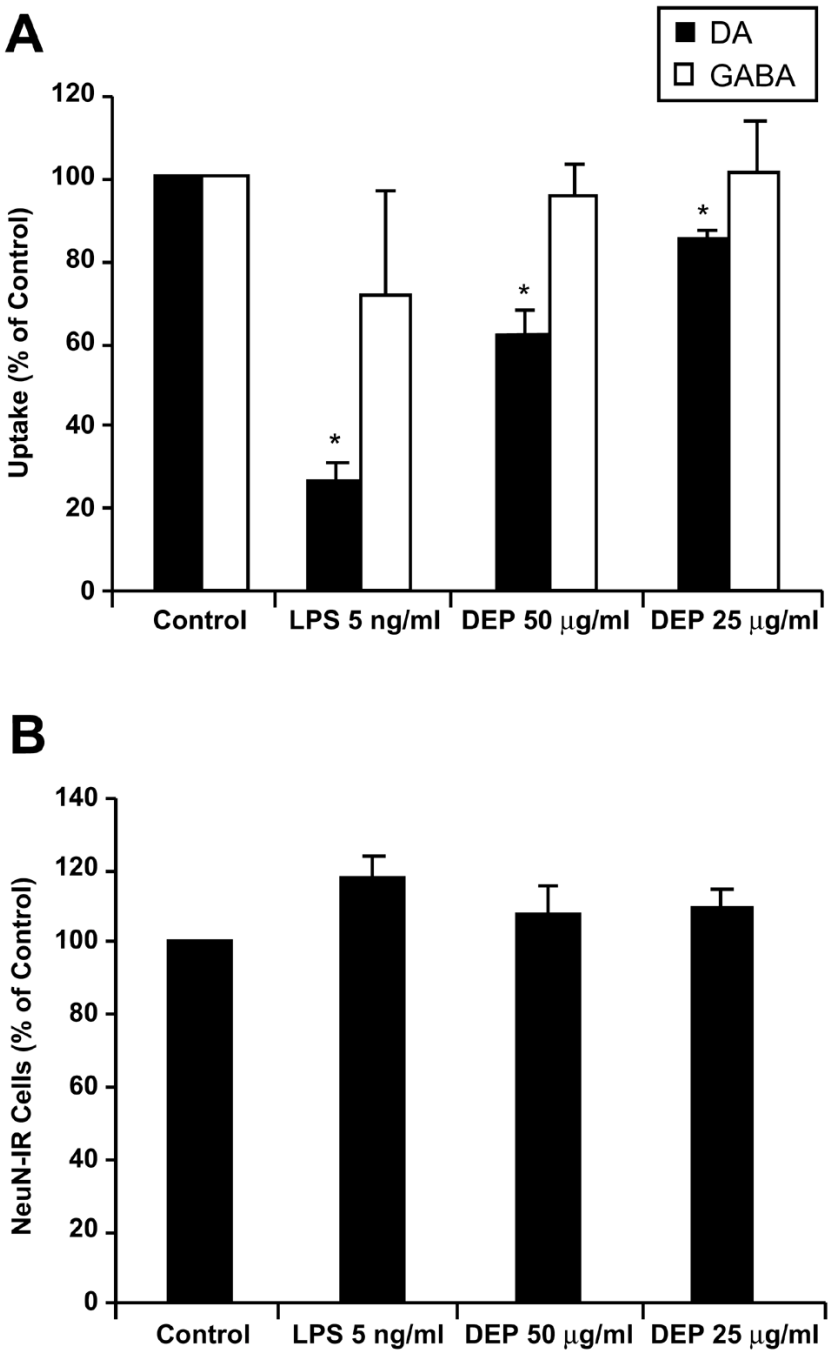

Figure 2. DEP neurotoxicity is specific for DA neurons. Mesencephalic midbrain neuron-glia cultures were treated with either vehicle, 5 ng/ml LPS, 50 μg/ml DEP, or 25 μg/ml DEP. LPS was used as a positive control for selective microglia-mediated DA neurotoxicity. ***A*)** DA neurotoxicity was measured at 8-9 days posttreatment using the [^{3}H]DA uptake assay and GABA neurotoxicity was determined using [^{3}H]GABA uptake assay. ***B*)** Effect of DEP on overall neuron number was determined at 8-9 days posttreatment with immunocytochemical staining using Neu-N antibody, specific for neuronal nuclear protein, and counting the number of Neu-N positive cells present. Data are percentage of control cultures, are means ± SE, and are the average of 3 separate experiments. *Significant difference (P<0.05) compared with control.

Fig. 3

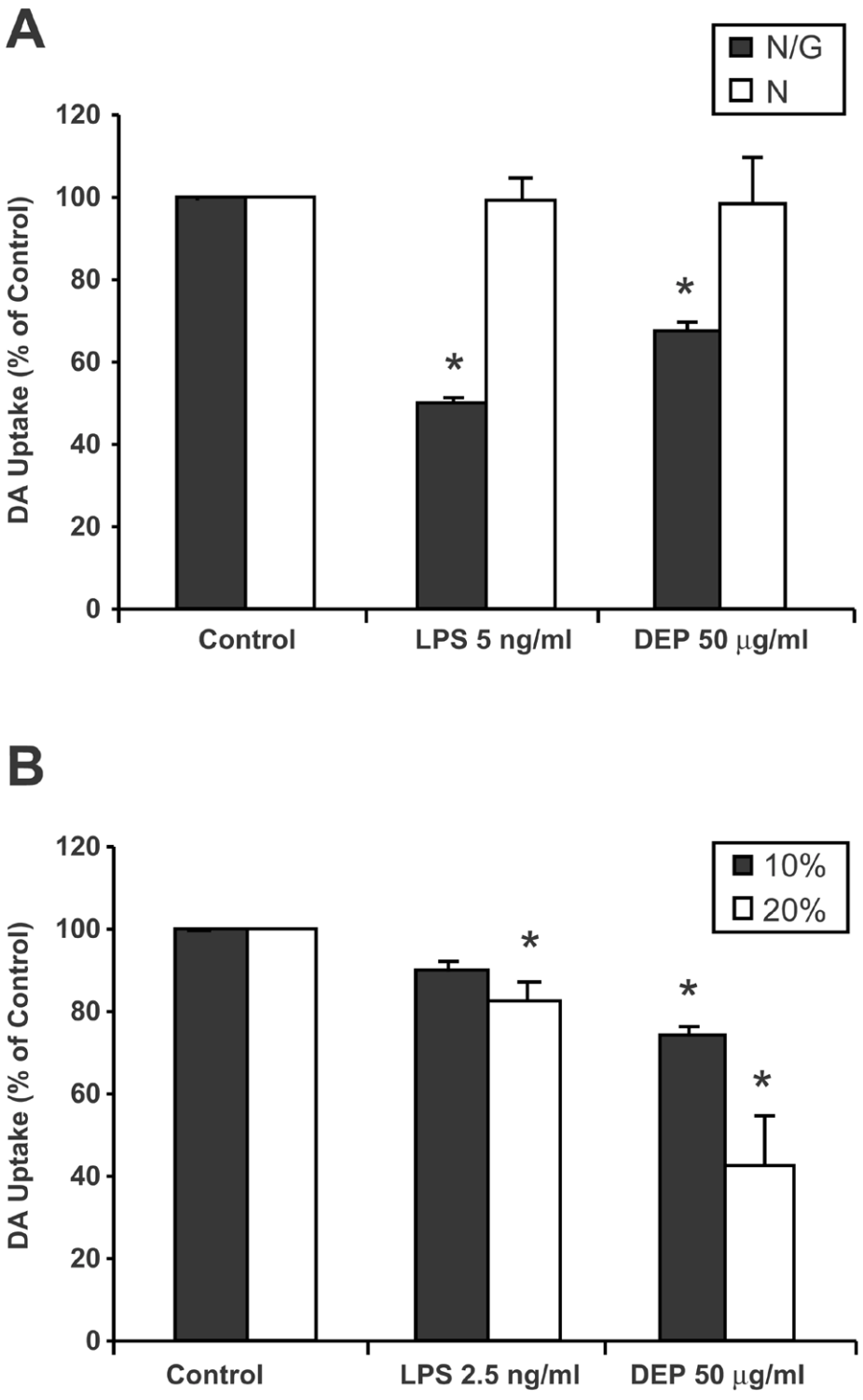

Figure 3. Microglia mediate DEP DA neurotoxicity. ***A*)** Mesencephalic midbrain neuron-glia cultures and neuron-enriched cultures were treated with either vehicle, 5 ng/ml LPS, or 50 μg/ml DEP. LPS was used as a positive control for microglia-mediated DA neurotoxicity. ***B*)** Microglia (10 and 20%) were added back to neuron-enriched cultures to test the importance of microglia to DEP DA neurotoxicity. DA neurotoxicity was measured at 8-9 days posttreatment using the [^{3}H]DA uptake assay. Data are percentage of control cultures, are means ± SE, and are the average of 3 separate experiments. *Significant difference (P<0.05) compared with control.

Fig. 4

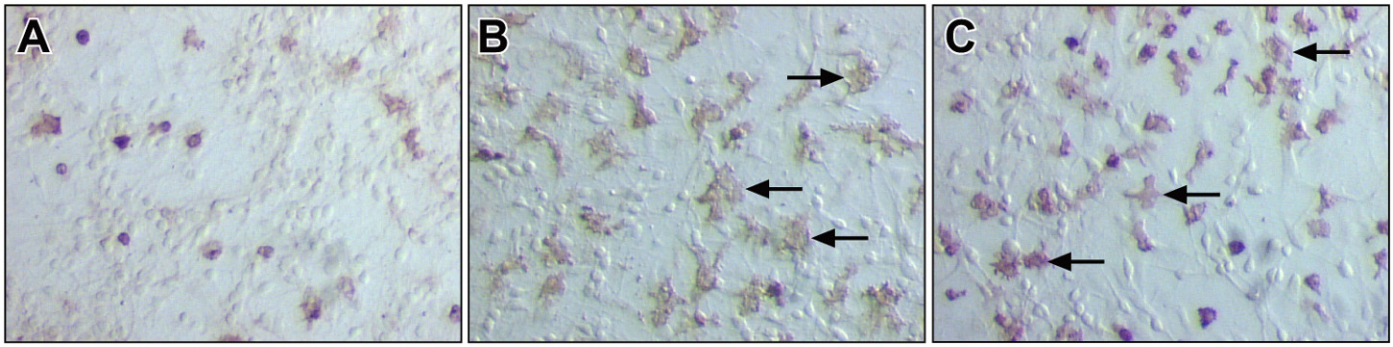

Figure 4. DEP induce proinflammatory morphology in microglia. Mesencephalic midbrain neuron-glia cultures were treated with either vehicle (***A***), 5 ng/ml LPS (***B***), or 50 μg/ml DEP (***C***). LPS was used as a positive control for microglia activation. Twelve hours posttreatment, cells were fixed and immunoassayed with an antibody to OX-42, a common microglial marker. Microglial activation (arrows) is depicted by increase in intensity of OX-42 immunostaining, increase in microglial size, and irregular and distorted morphology. Images are representative of 3 independent experiments.

Fig. 5

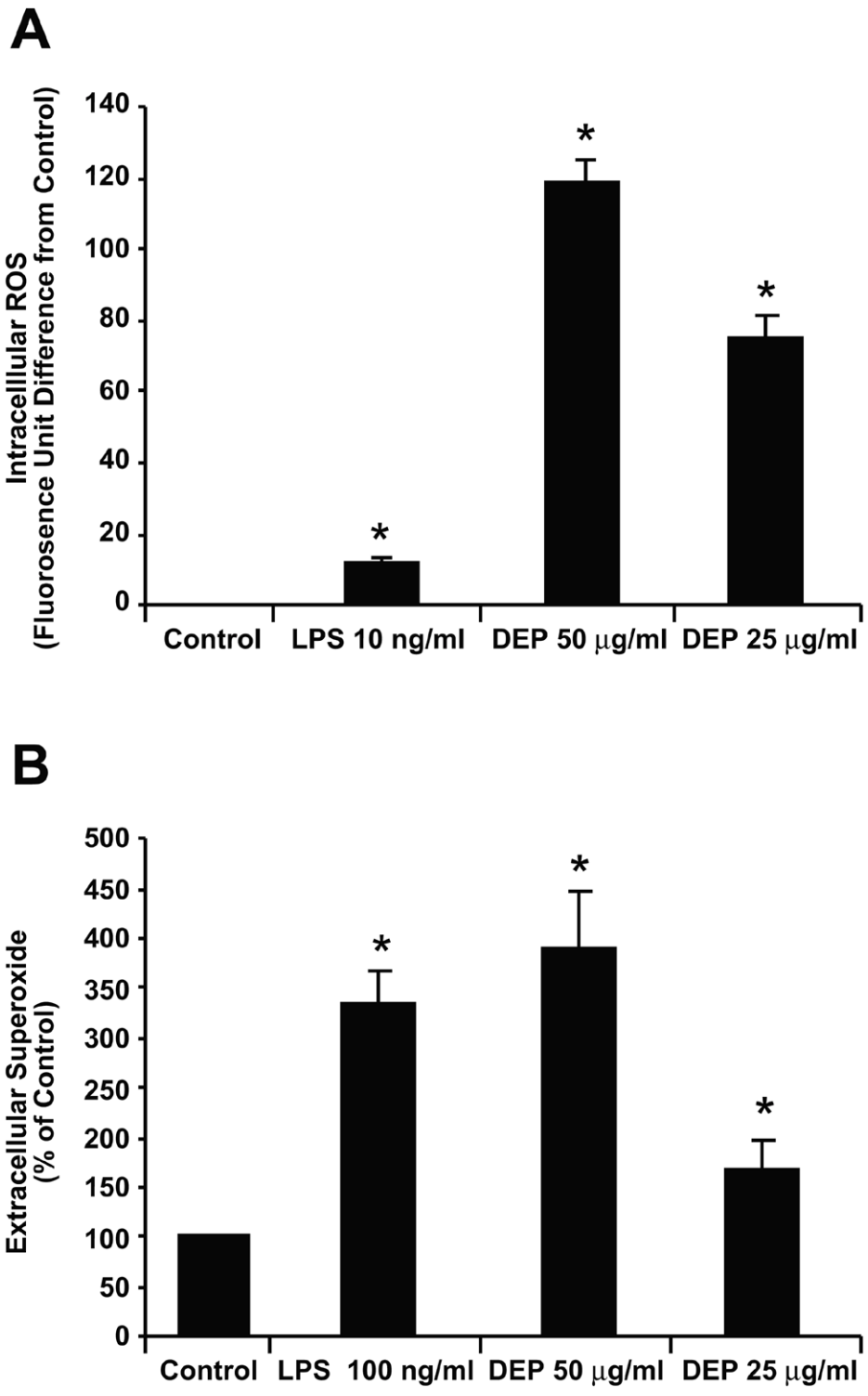

Figure 5. DEP induce production of reactive oxygen species (ROS) from microglia. LPS was used as a positive control for microglia activation. Enriched-microglia cultures were treated with either vehicle, LPS, 50 μg/ml DEP, or 25 μg/ml DEP. ***A*)** Production of intracellular ROS was measured 3 h poststimulation using DCFDA. ***B*)** Production of extracellular superoxide was measured by the superoxide dismutase (SOD) inhibitable reduction of tetrazolium salt, WST-1. Data are percentage of control cultures, are means ± SE, and are the average of 3 separate experiments. *Significant difference ($P<0.05$) compared with control.

Fig. 6

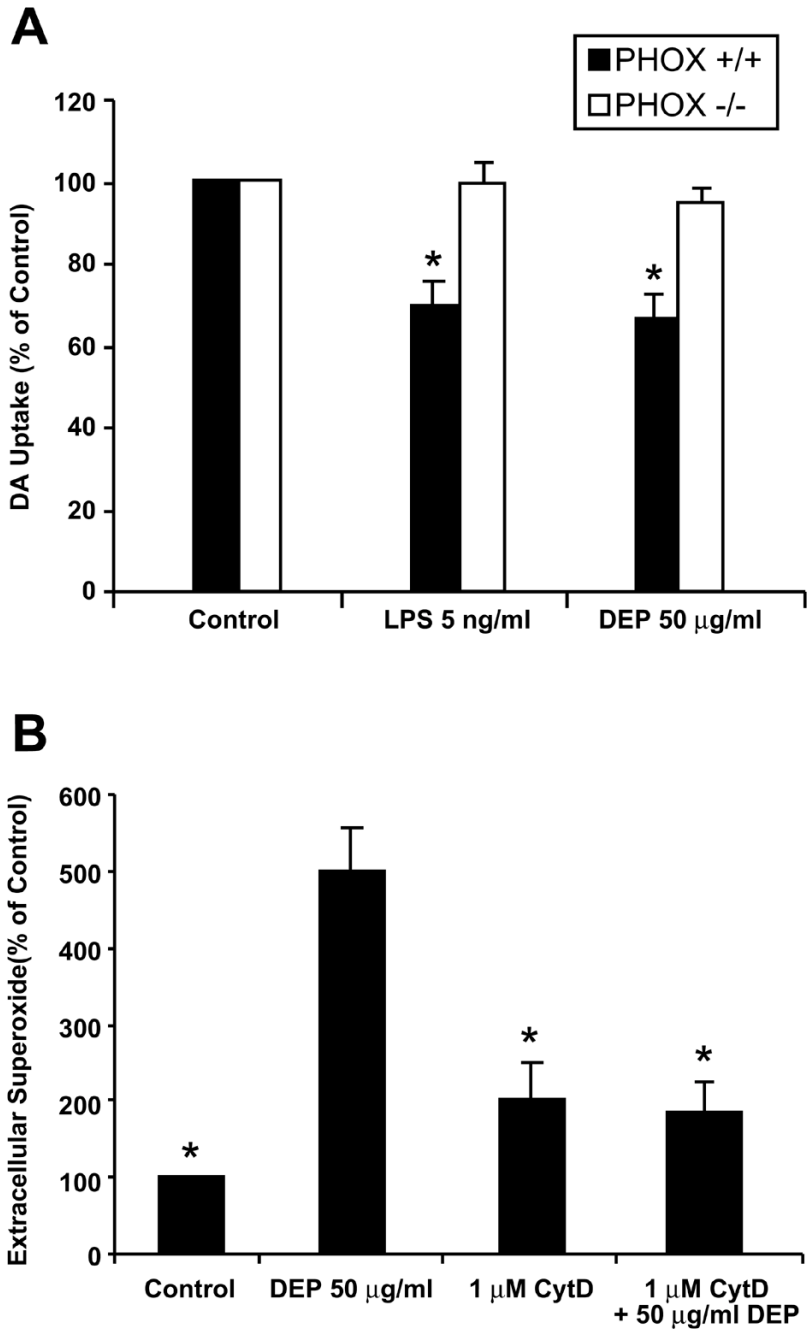

Figure 6. Role of NADPH oxidase and phagocytosis in DEP-induced DA neurotoxicity and oxidative insult. ***A*****)** NADPH oxidase mediates DEP DA neurotoxicity. PHOX$^{-/-}$ mice lack the functional catalytic subunit of NADPH oxidase complex, gp91 and fail to generate phagocytic respiratory burst. Mesencephalic midbrain neuron-glia cultures from PHOX $^{-/-}$ and PHOX $^{+/+}$ mice were treated with either vehicle, 5 ng/ml LPS, or 50 μg/ml DEP. LPS was used as a positive control for microglia-mediated DA neurotoxicity. DA neurotoxicity was measured at 8-9 days post treatment using the [^{3}H]DA uptake assay. Data are percentage of control cultures, are means ± SE, and are the average of 3 separate experiments. *Significant difference (P<0.05) compared with control. ***B*****)** Phagocytosis mediates DEP-induced superoxide production in microglia. Production of extracellular superoxide was measured by SOD inhibitable reduction of tetrazolium salt, WST-1. Preincubation with 1 μM cytochalsin D for 15 min inhibited DEP-induced super-oxide production in enriched microglia cultures. Data are percentage of control cultures, are means ± SE, and are the average of 3 separate experiments. *Significant difference (P<0.05) compared with DEP (50 μg/ml) treatment.

Fig. 7

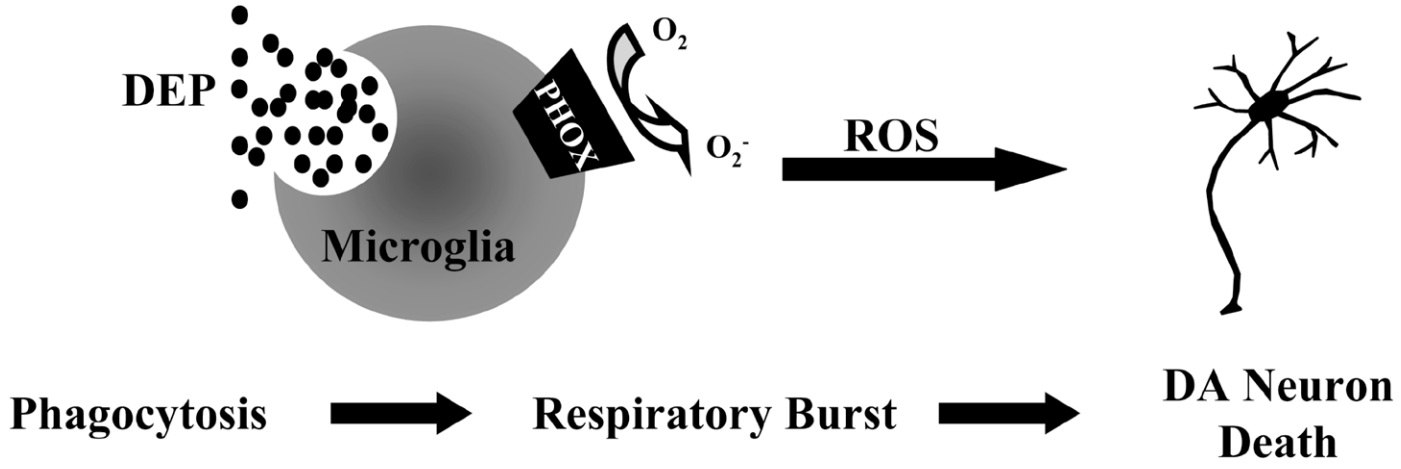

Figure 7. Phagocytosis-mediated DA neurotoxicity. DEP are phagocytized by microglia, which results in activation of NADPH oxidase (PHOX) and the neurotoxic respiratory burst. DA neurons are particularly vulnerable to oxidative damage and may have an increased sensitivity to ongoing phagocytosis from neighboring microglia compared with other neuronal cell types.