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Chemicals in Diesel Exhaust Particles Generate Reactive Oxygen Radicals and Induce Apoptosis in Macrophages

Timothy S. Hiura, Martin P. Kaszubowski, Ning Li, and Andre E. Nel

There is increasing evidence that particulate air pollutants, such as diesel exhaust particles (DEP), potentiate chronic inflammatory processes as well as acute symptomatic responses in the respiratory tract. The mechanisms of action as well as the cellular targets for DEP remain to be elucidated. We show in this paper that the phagocytosis of DEP by primary alveolar macrophages or macrophage cell lines, RAW 264.7 and THP-1, leads to the induction of apoptosis through generation of reactive oxygen radicals (ROR). This oxidative stress initiates two caspase cascades and a series of cellular events, including loss of surface membrane asymmetry and DNA damage. The apoptotic effect on macrophages is cell specific, because DEP did not induce similar effects in nonphagocytic cells. DEP that had their organic constituents extracted were no longer able to induce apoptosis or generate ROR. The organic extracts were, however, able to induce apoptosis. DEP chemicals also induced the activation of stress-activated protein kinases, which play a role in cellular apoptotic pathways. The injurious effects of native particles or DEP extracts on macrophages could be reversed by the antioxidant, N-acetyl-cysteine. Taken together, these data suggest that organic compounds contained in DEP may exert acute toxic effects via the generation of ROR in macrophages. The Journal of Immunology, 1999, 163: 5582–5591.

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Key scientific issues about the role of DEP that need to be addressed are: 1) what are the cellular targets? and 2) what are the mechanisms of action of DEP on those target cells? These are complicated issues due to the diversity of cell types that participate in airway inflammation as well as the chemical composition of DEP. Among the participating cell types in allergic inflammation, DEP or DEP extracts have been shown to affect eosinophil, epithelial, B cell, and macrophage function. However, we do not know whether these are direct or indirect effects. DEP are comprised of an inert carbonaceous core coated with unburnt petrochemicals. DEP contain >450 different organic compounds, including xenobiotics such as polycyclic aromatic hydrocarbons (PAH), halogenated aromatic hydrocarbons (HAH), and redox-active quinones. Xenobiotics, including PAH, exert stimulatory or toxic effects via the generation of reactive oxygen radicals (ROR) (24, 30–33). Macrophages, in particular, are enriched for xenobiotic converting enzymes, which contribute to ROR generation (1, 33, 34–38). Examples of these enzymes include cytochrome P4501A1, a phase I drug-metabolizing enzyme that is induced by PAH and HAH, as well as NADH-cytochrome P450 reductase, which is induced by PAH-derived chemicals. ROR generation has also been linked to the mutagenic effects of DEP chemicals (30) as well as to the activation of stress-activated protein kinases (SAPKs), which regulate the expression of proinflammatory genes in macrophages (24, 40). Based on these findings, we have proposed that macrophages are a key cellular target for DEP in the respiratory tract. Moreover, the targeting of macrophages may explain the adjuvant effects of DEP, e.g., enhancement of their Ag-presenting function. This process may involve ROR generation. In addition to their effects on cellular activation, ROR may play a role in the acute respiratory effects of PM10 pollutants.

One manifestation of the acute toxicity of DEP at the cellular level is the induction of apoptosis in macrophages. Xenobiotics can induce cellular apoptosis (41, 42) and may contribute to DEP-induced apoptosis. Macrophage apoptosis may impact tissue responses in two...
ways. First, the shedding of apoptotic bodies may spread toxic chemicals to neighboring cells, which engulf the apoptotic remnants from dying cells. Second, damage to macrophages may decrease tissue defenses to infection, e.g., increased multiplication of respiratory viruses in the lungs of mice exposed to DEP (43). Apoptosis may also be relevant to chronic airway inflammation (44), including COPD, where macrophages play a role in perpetuating inflammation in small airways (45). We were interested whether the proapoptotic effects of DEP are related to the ability of macrophages to phagocytose this particular matter, and whether the chemicals contained in DEP play a role in cellular toxicity. Our data show that apoptosis could be induced by phagocytosis of intact DEP or adding the organic extracts made from these particles back to the macrophage cell lines. In contrast, organically extracted DEP or carbon black particles did not induce apoptosis in macrophages. Apoptosis could be reversed by a caspase inhibitor as well as the antioxidant, N-acetyl-cysteine (NAC). This system provides us with a cellular model to study the role of oxygen radicals in DEP-related tissue injury.

Materials and Methods

Reagents

RPMI 1640 and FCS were purchased from Irvine Scientific (Santa Ana, CA). DMEM, penicillin-streptomycin, and L-glutamine were purchased from Life Technologies (Gaithersburg, MD). DEP were a gift from Dr. Masaru Sagai (National Institute of Environmental Studies, Tsukuba, Japan) (33). An E-Toxate kit, NAC, EDTA, and propidium iodide (PI) were purchased from Sigma (St. Louis, MO). Z-VAD was obtained from Enzymes Systems Products (Dublin, CA). The annexin-FITC kit was purchased from Trevigen (Gaithersburg, MD). 2′,7′-Dichlorofluorescein diacetate (DCF-DA) was purchased from Molecular Probes (Eugene, OR). Anti-caspase 8 and anti-caspase 9 Abs were obtained from PharMingen (San Diego, CA). Anti-phospho-JNK and anti-phospho-p38 MAPK Abs were purchased from Trevigen (Gaithersburg, MD). 2

Cell culture and stimulations

RAW 264.7 cells (murine) were cultured in a 5% CO2 atmosphere in DMEM containing 10% FCS, 1% penicillin-streptomycin, and 1% glutamine. THP-1 cells (human) were cultured under the same conditions, except that RPMI 1640 medium was used. Pulmonary alveolar macrophages, obtained by saline lavage of the lung of an adult Fisher rat, were obtained by Dr. J. Seagrave (Lovelace Respiratory Research Institute, Albuquerque, NM). In phagocytosis assays, aliquots 3 × 106 cells were cultured in six-well plates in 3 ml of medium. These cultures were replenished with sterilized fluorescent latex beads, native DEP, methanol-extracted DEP, or carbon black particles to final concentrations as indicated. To assess phagocytosis of the latex beads, cells were gently scraped from the plates 1 h later, washed three times in PBS, and fixed in 1% paraformaldehyde for 20 min on ice (46). Cells were then viewed under a fluorescence microscope. To assess the phagocytosis of DEP, washed DEP, or carbon black particles, cells were harvested after 18 h, washed, and viewed under a Kodak light optic microscope (Eastman Kodak, Rochester, NY) at a ×1000 magnification.

Preparation of DEP and washed DEP

A stock of DEP was prepared by resuspending 100 mg of DEP in 2 ml of PBS supplemented with 10% FCS. The particles were vortex mixed vigorously, sonicated with a sonic disruptor for 2 min on ice, and then stored at 4°C in the dark. Washed DEP were prepared as follows. DEP (100 mg) was suspended in 50 ml of methanol and vigorously vortex mixed (47). The sample was sonicated for 2 min on ice and centrifuged for 10 min at 2500 rpm. Methanol extracts were saved for later use. The DEP pellet was washed three times in 50 ml of methanol, dried, and reweighed to calculate the relative contribution of the extractable and nonextractable components to the overall particle composition. The sample was then resuspended in 10% FCS in PBS to yield a stock concentration of 50 mg/ml washed DEP. This stock was diluted in complete medium to the indicated concentration at the start of every experiment. DEP extracts were prepared for use in culture by aliquoting 1.5 ml of the methanol extract into weighed Eppendorf tubes. After methanol evaporation by nitrogen gas, the tubes were reweighed to calculate the extract amount. The dried extracts were resuspended in DMSO to give a stock concentration of 100 µg/ml DEP, washed DEP, and DEP extracts were tested to determine the absence of endotoxin using the E-Toxate kit.

Flow cytometry

Flow cytometric analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA) equipped with a single 488-nm argon laser. Dead cell fragments were gated out by forward and side scatter analysis. Annexin V-FITC and DCF were analyzed at the same excitation and emission settings of 488 and 535 nm, respectively (FL-1 channel). One-color PI analysis was performed at excitation and emission settings of 488 and 575 nm, respectively. Where dual color PI annexin V-FITC analysis was performed, PI fluorescence was displayed in channel FL-2, while the FITC fluorescence was displayed in channel FL-1.

PI staining

Cells (3 × 106) were plated into 3.5-cm plates in 3 ml of medium and rested for 4 h. Some cultures were preincubated with 20 mM NAC for 2 h. Varying concentrations of DEP or DEP extracts were added to the cultures for 18 h. Cells were then collected, washed twice in PBS, and resuspended in 500 µl of PBS. PI, suspended in PBS, was added to these samples to a final concentration of 0.5 µg/ml for 5 min.

Determining apoptosis by hypodiploid DNA detection

Aliquots of 3 × 106 RAW 264.7 cells were incubated with 200 µg/ml DEP for 18 h. Samples were then washed twice with PBS and resuspended in 300 µl of saline GM solution (6.1 mM glucose, 137 mM NaCl, 5.4 mM KCl, 1.1 mM Na2HPO4, 1.1 mM KH2PO4, and 5.1 mM EDTA). Cells were fixed in 700 µl of cold 100% ethanol and incubated at −20°C for 1 h. After fixation and washing, cells were resuspended in 1 ml of hypotonic DNA staining buffer (3.4 mM sodium citrate, 0.15 mM propidium iodide, 0.002% (w/v) RNase A, and 0.003% Triton X-100). The samples were then incubated at 4°C in the dark for 30 min and analyzed by flow cytometry.

Annexin V/PI staining

RAW 264.7 cells (3 × 106) were plated in six-well plates. Varying concentrations of DEP, washed DEP, or carbon black were then added to the cultures for 18 h. Cells, at a concentration of 1 × 106 cells/ml, were washed three times in PBS and resuspended in annexin V/PI staining buffer according to manufacturer’s instructions. After incubating at room temperature for 15 min, samples were analyzed by flow cytometry.

DCF staining

RAW 264.7 cells (3 × 106) were incubated together with 200 µg/ml DEP or 50 µg/ml DEP extract for the indicated time periods. Some cultures received 20 mM NAC for 2 h before the addition of DEP or DEP extracts. Cells were collected and washed three times in PBS. Samples were resuspended in DCF-DA diluted in DMEM to a concentration of 2.5 µM and incubated at 37°C for 30 min in the dark. PI was added to samples, which were immediately subjected to flow cytometric analysis.

Western blotting analysis to assess activation of caspases 8 and 9

THP-1 cells (3 × 106) were incubated together with 300 µg/ml DEP in the presence and the absence of 20 mM NAC for the indicated time periods. Cells were lysed in an SDS buffer, and 100 µg of lysate was separated by 10% SDS-PAGE and transferred to Immobilon-P membranes. Immunoblotting for caspase 8 was performed using a 1/500 dilution of the anti-caspase 8 Ab, followed by a 1/3000 dilution of HRP-coupled sheep antimouse Ab. Immunoblotting for caspase 9 was performed using a 1/1000 dilution of the anti-caspase 9 Ab, followed by a 1/3000 dilution of HRP-coupled donkey anti-rabbit Ab.

Phospho-JNK and phospho-p38 immunoblots

Immunoblots were performed by treating 3 × 106 THP-1 cells with 50 and 100 µg/ml DEP extracts or 10 µg/ml LPS. Cells were lysed as previously described, and 100 µg of lysate was separated by 10% SDS-PAGE. Blots were overlaid with a 1/1000 dilution of anti-phospho-JNK or anti-phospho-p38 Abs followed by a 1/3000 dilution of HRP-coupled donkey anti-rabbit Ab (1).
Results

RAW and THP-1 are phagocytic cell lines that ingest DEP

We have previously shown that PAH and redox-active quinones exert stimulatory as well as inhibitory effects in macrophages (1, 24). Because DEP contain similar organic chemicals, these particles may induce a variety of cellular responses in macrophages. Moreover, macrophages express xenobiotic converting enzymes such as cytochrome P4501A1, which plays a role in mediating the biological effects of PAH (34–38). To develop a relevant macrophage model for DEP phagocytosis, we used the murine RAW 264.7 and human THP-1 cell lines, which have previously been used to demonstrate the cellular effects of xenobiotics (1, 24). First, we had to establish that these cell types are phagocytic. For that purpose, we incubated THP-1 and RAW 264.7 cultures with fluorescent latex beads (46) and followed the cellular uptake of those beads by fluorescence microscopy and flow cytometry (Fig. 1). Fluorescence microscopy demonstrated that both cell types effectively phagocytosed latex beads (Fig. 1). In subsequent experiments, we looked at DEP phagocytosis under similar culture conditions. THP-1 and RAW 264.7 cells were incubated with different amounts of DEP for 18 h. After washing, the cells were viewed under a light optic microscope. Both cell types ingested DEP (Fig. 2). Taken together, the data in Figs. 1 and 2 show that RAW 264.7 and THP-1 cells can be used to study the cellular effects of DEP related to their phagocytosis.

DEP phagocytosis leads to cell death, which can be reversed by antioxidants or removal of organic compounds from the DEP

In the course of the DEP studies, we noticed the appearance of detached cells with blebbing surface membranes and apoptotic bodies in the culture supernatants. We used PI staining of the nucleus to determine whether these were dead cells. Fig. 3, A and B, demonstrates that adding increasing amounts of DEP led to a dose-dependent increase in the percentage of PI-stained cells in the

FIGURE 1. RAW 264.7 and THP-1 cell lines phagocyte fluorescent latex beads. Panels 1 and 3 are light optic photographs of RAW 264.7 and THP-1 cells incubated with 500 μg/ml Flurobrite beads for 18 h. Panels 2 and 4 represent the same fields under fluorescent light.

FIGURE 2. THP-1 and RAW 264.7 cells lines phagocyte DEP. Light optic microscopy showing THP-1 and RAW 264.7 cells before (panels 1 and 3) and after (panels 2 and 4) exposure to 200 μg/ml DEP for 18 h.
particles had an effect on their toxicity. After methanol extraction, reweighing, and adding the washed DEP particles to the cultures, it could be seen that there was a dramatic reduction in the rate of cell death (Fig. 3, A and B). This difference was not due to diminished phagocytosis, because the washed particles were equally well ingested as the native DEP (not shown). In addition, equal amounts of unwashed carbon black particles, which were readily phagocytosed, failed to exert toxic effects on THP-1 or RAW 264.7 cells (not shown). Taken together, these data show that the chemical composition of DEP is responsible for their toxicity.

DEP contain a wide range of chemicals, some of which are known inducers of ROR (30, 33). To determine the possible contribution of oxidative stress to DEP-induced cell death, both macrophage types were pretreated with the antioxidant, NAC, before coincubation with different doses of DEP. Using flow cytometry to measure PI staining of the nucleus as a measure of cell death, it could be seen that NAC effectively (>80%) inhibited the induction of cell death in RAW 264.7 cells at all DEP concentrations tested (Table II). While NAC reduced the rate of cell death in THP-1 cells by >60% at a lower (200 μg/ml) DEP concentration, this antioxidant had little effect on reversing cell death at the higher (400 μg/ml) particle concentration (Table II). This shows that while ROR play a role in the induction of cell death, additional factors may determine the susceptibility of THP-1 cells to DEP.

**Phagocytosis of DEP leads to apoptotic cell death**

It has recently been shown that biological responses to PAH and HAH include the induction of apoptosis (41, 42). Because these toxic chemicals are present in DEP, we asked whether the cellular events depicted in Figs. 3 and Table II reflect the induction of apoptosis. The morphological features of the dying macrophages in our cell culture were compatible with apoptosis (not shown). These apoptotic features included changes in the plasma membrane, chromatin condensation, disruption of the nucleus, and the formation of membrane-bound apoptotic bodies. The early loss of membrane asymmetry can be conveniently followed using annexin V staining, which detects aberrantly expressed phosphatidylserine on the outside of the cell membrane (48). This analysis can be combined with PI staining in a two-color flow cytometry protocol, which additionally reflects cell death. Exposure of RAW 264.7 cells to DEP for increasing lengths of time demonstrated a time-dependent increase in annexin V staining (Fig. 4A). Approximately 20% of the cells stained annexin V positive but remained PI negative after a 6-h incubation period (Fig. 4A, right lower quadrant). This fluorescence profile is compatible with early apoptotic events. However, by 18 h this profile changes to reflect the additional appearance of PI-positive cells in the cell population (Fig. 4A, right upper quadrant). Some annexin V-positive but PI-negative

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**Table I. Percentage apoptosis BEAS-2B cells after exposure to DEP**

<table>
<thead>
<tr>
<th>DEP (μg/ml)</th>
<th>Apoptotic Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.12 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>1.93 ± 0.3</td>
</tr>
<tr>
<td>25</td>
<td>3.37 ± 0.8</td>
</tr>
<tr>
<td>50</td>
<td>2.91 ± 0.1</td>
</tr>
<tr>
<td>100</td>
<td>4.61 ± 1.0</td>
</tr>
<tr>
<td>200</td>
<td>3.71 ± 1.3</td>
</tr>
</tbody>
</table>

*BEAS-2B cells were treated with DEP at indicated concentrations for 18 h. The percentage apoptotic cells were determined by assessing the hypodiploid DNA content after PI staining of nuclei. The results represent the mean of two separate experiments ± SEM.

**Table II. The effects of NAC on DEP-induced cell death (% dead cells)**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>DEP Concentration (μg/ml)</th>
<th>− NAC</th>
<th>+ NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAW 264.7</td>
<td>None</td>
<td>3.1</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>61.5</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>87.5</td>
<td>11.0</td>
</tr>
<tr>
<td>THP-1</td>
<td>None</td>
<td>2.0</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>55.2</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>72.1</td>
<td>67.6</td>
</tr>
</tbody>
</table>

* Aliquots of 3 × 10⁶ cells were treated with the indicated concentrations of DEP for 18 h in the presence and absence of 20 mM NAC. Cells were washed and stained with propidium iodide followed by flow cytometric analysis. The results represent the mean of two separate experiments in which SEM varied <10%.
cells could still be seen at this stage, indicating that new apoptotic events were occurring (Fig. 4A, right lower quadrant). Additional evidence for induction of apoptosis by DEP was obtained by assessing hypodiploid DNA in PI-stained RAW 264.7 cells (Fig. 4B). In the same analysis, which also reflects cell cycle progression, it could be seen that DEP induce cell arrest in the G1 phase of the cycle (Fig. 4B). Similar observations were made in THP-1 cells as well as primary alveolar macrophages (see below).

In a subsequent experiment, we compared the effects of different concentrations of native DEP with washed DEP or carbon black particles. Two-color flow analysis demonstrated that while washed DEP or carbon black particles failed to induce significant apoptosis, unwashed DEP did induce apoptosis at all concentrations tested (Fig. 5A). In a parallel experiment in which RAW 264.7 cells were incubated with washed DEP and carbon black particles, only marginally increased rates of apoptosis were recorded compared with the control values (Fig. 5A). Similarly, in THP-1 cells, DEP induced dose-dependent apoptosis (Fig. 5B). Although NAC effectively interfered with apoptosis at the lower (200 μg/ml) DEP concentration (18.4 vs 46.1%), this antioxidant had only a minor blocking effect (64 vs 47%) on the higher (400 μg/ml) DEP concentration. However, in RAW 264.7 cells, NAC was effective in decreasing apoptosis at all DEP concentrations tested (not shown). These data agree with the PI staining data in Table II.

A hallmark of apoptosis is the activation of the caspase family (49). These cysteine proteases play a pivotal role in the initiation and execution of cell death, including cell death induced by toxic chemicals and oxidative stress stimuli. Among the 10 or more members of the caspase family, caspase 9 activation has been linked to the mitochondrial release of cytochrome c (50). Moreover, ROR can initiate apoptosis through mitochondrial effects. To

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** DEP-induced cell death is an apoptotic process that can be reversed by extracting the particles with methanol. A. Two-color flow cytometry showing the kinetics of DEP-induced apoptosis. RAW 264.7 cells were treated with 300 μg/ml DEP for the indicated time periods. Cellular staining instrument settings and gating procedures are described in Materials and Methods. The results are representative of three independent experiments. B. Confirmation of apoptosis by determining hypodiploid DNA in PI-stained RAW 264.7 cells. Cells were treated with 200 μg/ml DEP for 18 h, followed by DNA ploidy analysis in PI-stained nuclei.

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Two-color flow cytometry to assess the effects of a DEP dose range and NAC on cellular apoptosis. A. Two-color flow analysis showing comparison of the effects of DEP at 200 and 400 μg/ml with equal amounts of methanol-extracted (washed) DEP and carbon black particles. The incubation time with RAW 264.7 cells was 18 h. B. Similar analysis showing the effect of 20 mM NAC in THP-1 cells coincubated with 200 and 400 μg/ml DEP for 18 h.
study the activation of this caspase, we used an immunoblotting technique that shows the cleavage of inactive procaspase 9 to activated caspase 9 (49). When THP-1 cells were exposed to 300 μg/ml DEP, we observed a time-dependent decrease in the staining intensity of the 46-kDa procaspase from 6 h onward (Fig. 6A). Moreover, the proteolysis of this procaspase yielded a 37-kDa fragment that represents the active caspase (Fig. 6A, lane 4). The time point for caspase 9 activation agrees with the time point for positive annexin V staining (Fig. 4A). The addition of NAC to THP-1 cells prevented caspase 9 activation (Fig. 6A, lanes 6–10), confirming the importance of oxidative stress in DEP-induced apoptosis.

In addition to a putative mitochondrial initiation pathway (50), there are other apoptosis initiation pathways. One possibility is the expression of membrane receptors that recruit procaspase 8 to an assembly of postreceptor proteins that express the death effector domain (51). Using an immunoblotting technique that looks at the cleavage of procaspase 8, we could observe DEP-induced proteolysis, starting from 6 h onward (Fig. 6B, lanes 1–5). As for caspase 9 activation, NAC effectively interfered with this proteolytic event (Fig. 6B, lanes 6–10).

Whatever the mechanism of initiation of these caspase cascades, the afferent caspases activate a series of downstream caspases that play a role in the execution of apoptosis (49). A broad spectrum caspase inhibitor, the fluoro-methyl ketone derivative, Z-VAD-fmk, inhibited DEP-induced apoptosis by >50% at a concentration of 30 μM (not shown). This confirms the importance of caspases in DEP-induced apoptosis.

### Table III. The effect of NAC on induction of RAW264.7 cell death

<table>
<thead>
<tr>
<th>DEP Extract (mg/ml)</th>
<th>None</th>
<th>30</th>
<th>60</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Dead Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>− NAC</td>
<td>3.8</td>
<td>10.3</td>
<td>42.8</td>
<td>74.7</td>
</tr>
<tr>
<td>+ NAC</td>
<td>4.2</td>
<td>6.0</td>
<td>12.1</td>
<td>13.2</td>
</tr>
</tbody>
</table>

A aliquots of 3 × 10⁶ RAW 264.7 cells were treated with the indicated concentrations of DEP extracts for 18 h. Cells were stained and PI followed by flow cytometric analysis. The results represent the mean of two separate experiments in which SEM varied <10%.

### Organic DEP extracts induce apoptosis in macrophage cell lines as well as primary alveolar macrophages

In Fig. 3 we have demonstrated that washed or organically extracted DEP lack the toxicity of native DEP. Using PI staining, we could demonstrate a dose-dependent increase in the rate of cell death when the dried methanol extracts, resuspended in DMSO, were added back to RAW 264.7 cultures (Table III). Thirty, 60, and 100 μg of extract represent 80, 160, and 250 μg DEP, respectively. Two-color flow cytometry confirmed that this is an apoptotic event (Fig. 7A). Moreover, the induction of apoptosis could be reversed by the antioxidant, NAC (Table III and Fig. 7A).

DEP and DEP extracts also induced programmed cell death in pulmonary alveolar macrophages obtained from a rat lung (Fig. 7B). Methanol extraction decreased the toxicity of the DEP (Fig. 7B). Taken together with the data in Fig. 3, we conclude that methanol-extractable DEP chemicals are responsible for apoptosis in primary macrophages as well as cell lines.

### DEP and DEP extracts generate ROS in an NAC-sensitive manner

Because DEP-induced apoptosis can be reversed by NAC, we studied ROS generation with the dye, DCF-DA (52). DCF-DA is a nonfluorescent compound that is freely taken up into cells, where it is trapped by removing the DA group. Upon interaction with peroxides, DCF is converted into a fluorescent product. Treatment of RAW 264.7 cells with 100 μg/ml of a DEP extract generated an almost 2 log-fold increase in mean DCF fluorescence intensity compared with that in untreated cells (Fig. 8A). The addition of 20 mM NAC diminished this response by >90% (Fig. 8A). Although native DEP exerted similar effects, methanol-extracted DEP did not generate a significant increase in DCF fluorescence (not shown). Increased DCF fluorescence could be observed within an hour of adding the DEP extract and peaked after 3 h (Fig. 8B). This is compatible with the latency of early apoptotic events (Fig. 5A). NAC effectively suppressed this response for the entire observation period (Fig. 8B).

### DEP extracts induce p38MAPK and Jun kinase (JNK) activation in THP-1 cells

Because DEP-induced apoptosis can be reversed by NAC, PAH and redox-active quinones activate stress-activated protein kinases (SAPKs) in RAW 264.7 and THP-1 cells by an oxidant-sensitive mechanism (24). Because these kinases are involved in the cellular response to stress, including oxidant stress, we were interested in whether DEP extracts were successful in activating these kinases. Activation of JNK isoforms and p38MAPK can be assessed by immunoblotting, which reveals the phosphorylation of these kinases upon activation (1, 24). Our data show that DEP extracts induced dose-dependent phosphorylation of two different JNK species, as well as p38MAPK (Fig. 9). These increases in phosphorylation were not due to
changes in the abundance of the kinase enzymes, because parallel immunoblotting for kinase protein showed equal staining intensity in control and treated samples (see insets at bottom of Fig. 9, A and B). Ten micrograms of SB 230580, a p38 MAPK inhibitor, decreased the rate of apoptosis during treatment of THP-1 cells with 100 μg/ml DEP extract by 22% (not shown). Unfortunately, we could not directly test the involvement of the JNK cascade in these apoptotic events due to the lack of a specific inhibitor. The contribution, if any, of these kinases to DEP-induced apoptosis remains to be determined.

Discussion

In this paper we show that the phagocytic uptake of DEP induces apoptosis in macrophage cell lines as well as pulmonary alveolar macrophages. This process could be partially reversed by a broad spectrum caspase inhibitor. Based on the findings that organically extracted DEP are devoid of toxicity, while the methanol extracts maintain the ability to induce apoptosis (Figs. 3 and 7), we conclude that DEP chemicals are responsible for initiating a programmed cell death pathway. The particulate nature of DEP may be important, however, for targeting phagocytic cells (Fig. 2). Although the exact mechanism for the initiation of apoptosis remain to be elucidated, ROR play an integral role in this event (Fig. 8), as demonstrated by the ability of an antioxidant, NAC, to suppress the pro-apoptotic effects of DEP or DEP extracts (Figs. 5 and 7). This idea is further substantiated by the ability of NAC to inhibit DEP-induced generation of ROS (Fig. 8). In addition to the generation of oxidative stress, DEP extracts activated the stress-activated protein kinases, JNK and p38 MAPK, which may contribute to the cellular response to oxidative stress (Fig. 9). These findings have important implications for treating the effects of particulate pollutants on airway inflammation.

DEP is a major component of PM10 pollutants. For instance, in the Los Angeles basin, DEP comprise 40% of total PM10 levels, and the daily rate of exposure may be as high as 300 μg DEP (4). This dose is equivalent to what was used in human nasal challenge studies to demonstrate the adjuvant effects of DEP on allergic inflammation. Considering the limited surface area of the nose, a 300-μg DEP dose may deliver the same number of particles per unit mucosal area as to the lawn of cells in the culture dish. Moreover, even if a single in vivo exposure is not sufficient to achieve

FIGURE 7. DEP extracts induce apoptosis in RAW 264.7 as well as in rat pulmonary alveolar macrophages. A. Two-color flow showing the effect of 20 mM NAC on induction of apoptosis in RAW 264.7 cells exposed to organic extracts obtained from 200 μg/ml DEP. B. Two-color flow showing the effect of DEP, methanol-extracted (washed) DEP, and the organic DEP extract on primary alveolar macrophages obtained from a Fisher rat by a saline lavage procedure. Cell were exposed to 400 μg/ml DEP or 200 μg/ml DEP extract for 18 h.

FIGURE 8. DCF fluorescence showing that DEP extracts generate ROS in an NAC-sensitive manner. A. RAW 264.7 cells were treated with 100 μg/ml DEP extract in the presence or the absence of 20 mM NAC for 1.5 h. DCF fluorescence was determined as described in Materials and Methods. B. DCF fluorescence showing the kinetics of DEP extract-induced ROS activation in RAW 264.7. Cells were incubated with 100 μg/ml DEP extracts for the indicated time periods. Data represent the mean fluorescence intensity ± SEM of duplicate measurements.
54-kDa JNK isoforms by DEP extracts. Cells were treated with 50 and 100 μg/ml DEP extracts or 10 μg/ml LPS for 1 h. Blots were developed as described in Materials and Methods. The top panel represents a composite of the same blot with an Ab recognizing the kinase protein. B, Phospho-p38 MAPK immunoblotting showing activation of p38 by DEP extracts. Cellular stimulation was described in A. The bottom panel is a protein immunoblot.

FIGURE 9. DEP extracts induce p38MAPK and Jun kinase activation in THP-1 cells. A, Phospho-JNK immunoblot showing activation of 45- and 54-kDa JNK isoforms by DEP extracts. Cells were treated with 50 and 100 μg/ml DEP extracts or 10 μg/ml LPS for 1 h. Blots were developed as described in Materials and Methods. The bottom panel represents a composite of the same blot with an Ab recognizing the kinase protein. B, Phospho-p38 MAPK immunoblotting showing activation of p38 by DEP extracts. Cellular stimulation was described in A. The bottom panel is a protein immunoblot.

The relatively small size of DEP also allows their deposition in the lung and may be instrumental in the increased morbidity and mortality in people with asthma and COPD, which follow when there are sudden surges in PM10 levels (20, 21). Until recently, we had little understanding of the pathogenicity of DEP in the respiratory tract, but have now obtained evidence that at least two independent, but possibly interrelated, processes are at work. The first process is the adjuvant effects of DEP on allergic inflammation, as reflected in both human and animal studies (1–4, 13–19). Examples of such adjuvant effects include enhanced IgE and Th2 cytokine production during nasal challenge with an allergen in the presence of DEP (13). We have recently shown that macrophages may be involved in this adjuvant effects, based on the ability of DEP to increase CD86 expression in the nose in vivo and in macrophages in vitro (1). CD86 expression may enhance the properties of the macrophage as an APC (1). Longer term, these adjuvant events may play a role in the increased prevalence of asthma and allergic rhinitis, such as has been observed in Japan since the introduction of the diesel engine (11).

The second mechanism of action of DEP appears to be acute injurious events, such as the apoptotic events described in this paper. These events may be relevant to the acute symptomology and mortality that follow a rise in PM10 levels (20, 21). We postulate that ROR generation by macrophages may contribute to these acute symptomatic flares in asthmatics and people with COPD. The occurrence of apoptosis in macrophages may be useful as a marker for the acute injurious effects of DEP. It is also possible that macrophage apoptosis may directly contribute to the pathogenesis of respiratory disease. Although macrophage apoptosis has not been systematically studied in asthma, there are a limited number of studies that indicate that apoptosis pathways are relevant to allergic inflammation (44). We propose a number of ways in which macrophage apoptosis may contribute to chronic inflammation or perturbation of immune defenses in the lung. One consequence of macrophage cell death may be a decreased phagocytic capability, as has been shown in pulmonary alveolar macrophages after DEP ingestion (47). This interference in macrophage function may be responsible for an increased propensity toward respiratory tract infection, e.g., increased multiplication of influenza virus in DEP-exposed mice (43). Another contribution by macrophage apoptosis may be the shedding of apoptotic bodies when these cells die. Because these apoptotic bodies probably contain active chemicals, their uptake by surrounding inflammatory cell types may spread the tissue damage. We are particularly interested in whether this putative Trojan horse effect may induce epithelial cell damage, a prominent cause of increased bronchial hyper-reactivity in people with asthma. We are currently studying that possibility, including the effects of ROR being generated in macrophages on epithelial cells.

How do DEP induce toxic levels of ROR in macrophages? Although phagocytosis-related enzyme systems, such as membrane NADPH-oxidase, can contribute to ROR generation (53), macrophages are generally protected from their own respiratory burst activity. This explains the lack of an apoptotic effect by carbon black particles or organically extracted DEP (Fig. 5). Instead, native DEP or organic extracts made from those particles are effective in inducing apoptosis (Figs. 4–7). This suggests that DEP chemicals are responsible for generating the additional oxidant stress levels that are required to initiate apoptosis. Although these DEP-associated chemicals need to be identified, we know that DEP contain polycyclic aromatic compounds, halogenated aromatic hydrocarbons, and redox-active quinones (22, 27–29), all known inducers of ROR (24, 30–33) and apoptosis (41, 42). ROR generation by these chemicals involves drug-metabolizing enzymes such as microsomal p450 reductase and quinone reductases (33). In this regard, Kumagai et al. have demonstrated that the interaction of DEP-derived chemicals with NADPH-cytochrome p450 reductase generates reactive oxygen species (33). The same group demonstrated that DEP extracts induce superoxide and hydroxyl radical generation upon coinubcation with lung microsomal preparations (33). Because macrophages express xenobiotic converting enzymes, it is likely that a major source of ROR generation in the DEP-exposed lung is macrophages. Our data using DCF fluorescence confirm that DEP and DEP extracts generate ROR in macrophages (Fig. 8). Although we lack data about the specific chemicals in DEP extracts that are responsible for this effect, we do know that this is rapid event that peaks within 3 h (Fig. 8B). Moreover, this event appears to be specific for macrophages, because we did not find any evidence for ROR generation in a bronchial epithelial cell line, BEAS-2B, coincubated with DEP (Table...
The selective targeting of macrophages depends on the ability of these cells to ingest and concentrate DEP in the cell. At least one other group has demonstrated generation of oxidant stress by particulate pollutants in alveolar macrophages (54).

There are at least two major caspase cascades that play a role in apoptosis, one commencing with the activation of caspase 8 and the other with caspase 9 (49–51). Caspase 8 is activated by membrane receptors that recruit proteins with death effector domains (49), while procaspase 9 is activated by a mitochondrial released protein, cytochrome c (50). The ability of DEP to induce both caspasins may reflect distinct initiation pathways (Fig. 6). It is known, however, that some membrane receptors that activate caspase 8 may induce cytochrome c release, and it is possible, therefore, that caspase 8 activation may lead to caspase 9 activation. Although we still have to obtain evidence for mitochondrial involvement in our in vitro culture system, ROR generation has been shown to induce lipid peroxidation and protein oxidation in mitochondrial membranes (50). This may lead to cytochrome c release, which in the presence of the cofactor, Apaf-1, will induce procaspase 9 activation (49). This idea fits with our observations that ROR generation precedes the onset of apoptosis in RAW 274.7 and THP-1 cells (Figs. 4a and 4b). Moreover, the inhibitory effect of NAC on cells agrees with observations that NAC disrupts stress-induced apoptosis in other cell types (55). We are in the process of investigating the role of mitochondria in DEP-induced apoptosis. Regarding the role of receptor-mediated apoptosis pathways, it is interesting that the stress-activated protein kinase (JNK) plays a role in Fas ligand and TNF-α expression (56, 57). However, we did not observe any reduction in DEP-induced apoptosis using the Fas-Fc recombinant protein, which interferes with Fas ligand binding to Fas (not shown). There was also no significant effect of the DEP extract on TNF-α production, arguing against a role of the p55 TNF receptor (57). We did, however, observe a small decrease in the rate of apoptosis in the presence of a p38MAPK inhibitor, but it is not clear whether this inhibitory effect involves a receptor-mediated death event. There is no direct way to explore the involvement of JNK cascade in DEP-induced apoptosis at present.

Elucidation of target cells and the mechanism of action of DEP in the respiratory tract are of considerable clinical importance. The demonstration, for instance, that NAC effectively inhibited DEP-induced ROR generation and apoptosis induction predicts that antioxidants may be helpful in alleviating the effects of particulate pollutants in diseases such as asthma and COPD. Although a number of oral antioxidants are available, it may be useful to consider specific intrabronchial drug formulations that will target macrophages. For instance, it has been demonstrated that polyethylene glycol-aggregated superoxide dismutase, instilled intratracheally, reverses DEP-induced airway hyper-reactivity in mice (58).

In summary, we have elucidated a toxic cellular response that may explain the adverse effects of DEP in the respiratory tract. We predict that this type of cellular response may also be applicable to understanding the mechanisms of action of other toxic environmental pollutants in the lung.

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References