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# The Role of a Mitochondrial Pathway in the Induction of Apoptosis by Chemicals Extracted from Diesel Exhaust Particles<sup>1</sup>

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We are interested in the cytotoxic and proinflammatory effects of particulate pollutants in the respiratory tract. We demonstrate that methanol extracts made from diesel exhaust particles (DEP) induce apoptosis and reactive oxygen species (ROS) in pulmonary alveolar macrophages and RAW 264.7 cells. The toxicity of these organic extracts mimics the cytotoxicity of the intact particles and could be suppressed by the synthetic sulfhydryl compounds, *N*-acetylcysteine and buccillamine. Because DEP-induced apoptosis follows cytochrome *c* release, we studied the effect of DEP chemicals on mitochondrially regulated death mechanisms. Crude DEP extracts induced ROS production and perturbed mitochondrial function before and at the onset of apoptosis. This mitochondrial perturbation follows an orderly sequence of events, which commence with a change in mitochondrial membrane potential, followed by cytochrome *c* release, development of membrane asymmetry (annexin V staining), and propidium iodide uptake. Structural damage to the mitochondrial inner membrane, evidenced by a decrease in cardiolipin mass, leads to O<sub>2</sub><sup>-</sup> generation and uncoupling of oxidative phosphorylation (decreased intracellular ATP levels). *N*-Acetylcysteine reversed these mitochondrial effects and ROS production. Overexpression of the mitochondrial apoptosis regulator, Bcl-2, delayed but did not suppress apoptosis. Taken together, these results suggest that DEP chemicals induce apoptosis in macrophages via a toxic effect on mitochondria. *The Journal of Immunology*, 2000, 165: 2703–2711.

There is extensive epidemiological evidence that short-term exposure to elevated levels of particulate pollutants is associated with cardiopulmonary morbidity and mortality (1–5). Although the underlying mechanisms for these adverse health effects are largely unknown, it has been suggested that particulate matter (PM)<sup>4</sup> may induce oxidative lung damage and inflammation (5). This idea is consistent with the observed lag period between the surge in levels of PM with an aerodynamic diameter of 10 microns or less and the onset of symptoms (1–5).

Further support for the inflammation hypothesis comes from animal studies in which exposure to a model particulate pollutant, diesel exhaust particles (DEP), has been shown to induce acute pulmonary inflammation and injury (6, 7). DEP instillation into the trachea of mice leads to acute cytotoxic injury at the alveolar level, including degenerative changes of type I alveolar pneumocytes and capillary endothelial cells (7). This acute injury is followed by an intense inflammatory process in the lung and is associated with a high mortality rate (6, 7). The use of carbon black particles as a control in the same experiment was without the toxicity of DEP (7). Although DEP and carbon black particles both contain a carbon core, the former particle type contains an abundance of unburnt petrochemicals, which has led to the suggestion that the organic chemical components in DEP are responsible for tissue injury and inflammation (8–12).

Although the identity of DEP xenobiotics leading to acute lung injury are unknown, organic extracts made from DEP generate reactive oxygen species (ROS) in microsomes made from lung tissue (12). ROS are also involved in the toxic and proinflammatory effects of DEP in mice in vivo (6, 13, 14). ROS generation in microsomes was dependent on cytochrome P450 reductase activity, and the effects of the DEP chemicals in this assay can be inhibited by NaBH<sub>4</sub>, which reduces quinones to hydroxy derivatives (12). This suggests that quinones or oxidized polycyclic aromatic hydrocarbon (PAH) derivatives are responsible for ROS generation (12). Among the cell types that participate in ROS production in response to PM deposition in the lung is the macrophage (15–19). We are particularly interested in the role of macrophages in PM-induced inflammation for the following reasons: 1) macrophages are the principal cells that remove DEP and other PM from the bronchoalveolar region of the lung (20, 21); 2) macrophages phagocytose DEP, leading to the accumulation of toxic intracellular DEP concentrations (19); and 3) macrophages are endowed

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<sup>4</sup> Abbreviations used in this paper: PM, particulate matter; DEP, diesel exhaust particles; DiOC<sub>6</sub>, 3,3'-dihexyloxycarbocyanine iodide; DCF-DA, 2',7'-dichlorofluorescein diacetate; HE, hydroethidine; NAC, *N*-acetylcysteine; NAO, 10 *N*-nonylacridine orange; PI, propidium iodide; PAH, polycyclic aromatic hydrocarbon; PCD, programmed cell death; PT, permeability transition; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; tet, tetracycline;  $\Delta\psi_m$ , mitochondrial membrane potential; PAM, pulmonary alveolar macrophages; tTA, tet transactivator; hbc1-2, human bcl-2; SH, sulfhydryl.

with enzymatic pathways for ROS generation, including inducible cytochrome P450-dependent pathways (22–24).

We have recently shown that phagocytosis of DEP leads to programmed cell death (PCD) in macrophages (19). The same response is induced by an organic DEP extract and is suppressed by a synthetic sulfhydryl (SH) antioxidant, suggesting that the oxidative effects of DEP chemicals are involved in apoptosis (19). A key question is what are the intracellular targets for DEP-induced ROS generation and cell death. Mitochondria have recently received a lot of attention for their involvement in apoptosis induction and apoptosis-related ROS generation (25–32). These mitochondrial perturbations involve indirect or direct effects on the permeability transition (PT) pore (25–29). The PT pore is comprised of a complex assembly of inner- and outer-membrane proteins that regulate mitochondrial membrane permeability changes, ROS production, and the release of apoptogenic factors, such as cytochrome *c* (25–32).

The aim of this study was to investigate whether organic chemicals extracted from DEP induce PCD via a mitochondrially mediated pathway. In addition, we wanted to assess the role of ROS in these events and whether antioxidants and Bcl-2 can reverse the cytotoxic effects of DEP chemicals. Our data demonstrate that macrophage exposure to a methanol extract of DEP induces an orderly sequence of events in which the mitochondrial membrane perturbation leads to a decrease in mitochondrial membrane potential ( $\Delta\psi_m$ ), cytochrome *c* release, structural damage to mitochondrial inner membrane,  $O_2^-$  production, and uncoupling of oxidative phosphorylation. These results indicate DEP toxicity involves a mitochondrial pathway and ROS production.

## 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 (Baltimore, MD). pUHD10–3-bcl-2 and pTRE-hygro (hygromycin resistance element) were generously provided by Dr. Sarah Cunningham (Clontech Laboratories, Palo Alto, CA). An E-Toxate kit, N-acetylcysteine (NAC), EDTA, propidium iodide (PI), pyrrholidine dithiocarbamate (PDTC), ascorbic acid, silibinin, luteolin, and zymosan were purchased from Sigma (St. Louis, MO). The annexin-FITC kit was purchased from Trevigen (Gaithersburg, MD). Hydroethidine (HE), 2',7'-dichlorofluorescein diacetate (DCF-DA), 3,3'-dihexyloxycarbocyanine iodide (DiOC<sub>6</sub>), 10 N-nonylacridine orange (NAO), and an ATP measurement kit were purchased from Molecular Probes (Eugene, OR). Anti-cytochrome *c* Abs were purchased from R&D Systems (Minneapolis, MN). Six-well plastic tissue-culture dishes were purchased from Fisher Scientific (Pittsburgh, PA). Monoclonal anti-Bcl-2 Ab was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Normal mouse serum was purchased from Atlanta Biologicals (Atlanta, GA). Bucillamine was provided by Key-son Biomedical (Los Angeles, CA).

### Cell culture and stimulations

RAW 264.7 cells were cultured in a 5% CO<sub>2</sub> atmosphere in DMEM containing 10% FCS, 5000 U/ml penicillin, 500 µg/ml streptomycin, and 2 mM L-glutamine. Rat pulmonary alveolar macrophages (PAM) were obtained from male Fischer rats by bronchoalveolar lavage as previously described (19). The cell yield was typically  $5\text{--}7 \times 10^6$  cells/animal with >97% of the cells being macrophages. These cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine, penicillin-streptomycin, and 10% FBS. For exposure to DEP extracts, aliquots of  $3 \times 10^6$  cells were cultured in six-well plates in 3 ml of medium at 37°C for the indicated time periods. Some cultures received 20 mM NAC, 0.1–1 mM bucillamine, and 25–100 µg/ml each of PDTC, ascorbic acid, silibinin, or luteolin for 2 h before the addition of DEP extracts. Cells were harvested by scraping, washed three times in PBS, and then subjected to staining with fluorescent dyes.

### Source and preparation of DEP extracts

DEP collected from a light-duty diesel source were obtained from Dr. Masura Sagai (National Institute of Environmental Studies, Tsukuba, Ibaraki,

Japan) (6, 7, 12, 13). DEP extracts were prepared as previously described (19). Briefly, 100 mg of DEP were suspended in 50 ml of methanol and vigorously vortexed. The sample was sonicated for 2 min on ice and centrifuged for 10 min at 2500 rpm. Methanol extracts (1.5 ml) were aliquoted into a weighed Eppendorf tube. After methanol evaporation under nitrogen gas, the tubes were reweighed to determine the extract amount. The methanol-extractable component comprised 36% of the dry particle weight. The dried extracts were resuspended in DMSO to give the stock concentrations of 100 µg/µl. The stocks were stored in the dark at –20°C until use. DEP extracts were tested for endotoxin using the E-Toxate kit according to the manufacturer's recommendation (Sigma). All of our extracts were essentially endotoxin free.

### Cellular staining with fluorescent probes before performance of flow cytometry

Washed cells were stained with the fluorescent dyes at a concentration of 10<sup>6</sup> cells/ml. Except for annexin V and PI, the dyes were diluted in either DMEM or RPMI 1640. The combinations and final concentrations of the dyes are as follows: 1) 1 µl of annexin V plus 10 µl PI in 500 µl binding buffer supplied by the manufacturer (for assessment of early and late apoptotic events, respectively; Ref. 19); 2) 20 nM DiOC<sub>6</sub> plus 2 µM HE (for assessment of  $\Delta\psi_m$  and predominant  $O_2^-$  production, respectively; Ref. 33); 3) 2.5 µM DCF-DA plus 2 µM HE (for assessment of H<sub>2</sub>O<sub>2</sub> and  $O_2^-$  generation, respectively; Ref. 34); and 4) 100 nM NAO and 2 µM HE (assessment of cardiolipin mass and  $O_2^-$ , respectively; Ref. 35). Cells were incubated with these dyes for 15 min (procedure 1) or 30 min (procedures 2–4) at 37°C in the dark.

### Flow cytometry

Flow cytometric analysis was performed using a FACScan (Becton Dickinson, Mountain View, CA) equipped with a single 488-nm argon laser. DiOC<sub>6</sub>, NAO, annexin V-FITC, and DCF were analyzed using excitation and emission settings of 488 nm and 535 nm (Fl-1 channel). PI was analyzed using excitation and emission settings of 488 nm and 575 nm (Fl-2 channel). HE was analyzed using excitation and emission settings of 518 nm and 605 nm (Fl-3 channel). Forward and side scatter were used to gate out cellular fragments.

### ATP determination assay

Cells ( $3 \times 10^6$ ) were plated into 3.5-cm plates in 3 ml of media and rested for 4 h. Some cultures were preincubated with 20 mM NAC for 2 h. DEP extracts (100 µg/ml) were added to these cultures for varying amounts of time. Cells were collected, washed twice in PBS, and lysed in 200 µl H<sub>2</sub>O<sub>2</sub>. Samples were then boiled for 5 min and centrifuged at 14,000 rpm for 10 min. A Bradford assay was performed as previously described, and 500 ng protein was used to measure ATP content using a luciferin-luciferase ATP determination kit (Molecular Probes). The reagents were prepared according to the manufacturer's recommendations and read in a Monolight 2010 luminometer. ATP concentration was calculated according to the manufacturer's instructions.

### Western blotting analysis to assess mitochondrial release of cytochrome *c*

RAW 264.7 cells ( $3 \times 10^6$ ) were incubated together with 100 µg/ml DEP extract for the indicated time periods. Cells were lysed by three cycles of freeze-thawing in a lysis buffer containing 20 mM HEPES/KOH (pH 7.5), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 1 mM NaEDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose. Cells were then spun in a Beckman Coulter Ultracentrifuge (Fullerton, CA) at 100,000 × *g* (45,000 rpm) at 4°C for 30 min. The supernatant (S-100 fraction) was collected and protein concentrations were measured by the Bradford method. One hundred micrograms of lysate was separated by 5–20% gradient SDS-PAGE and transferred to Immobilon-P membranes as previously described (19). Immunoblotting for cytochrome *c* was performed using a 1:500 dilution of the anti-cytochrome *c* Ab, followed by a 1:3000 dilution of HRP-coupled sheep anti-mouse Ab.

### Transfection and generation of stable cell lines

A subclone of RAW 264.7, which stably expresses the tetracycline transactivator (tTA) protein (36), was a generous gift from Dr. Jawed Alam (Louisiana State University Medical Center, New Orleans, LA). RAW-tTA cells were transfected by electroporation using 20 µg of the tetracycline(tet)-repressible vector pUHD10–3 (31), into which human *bcl-2* (*hbcl-2*) was subcloned, together with 8 µg pTRE-hygro (hygromycin resistance element) as previously described (37). Cells were selected in 150

$\mu\text{g/ml}$  hygromycin for 3 wk. Individual colonies were picked and transferred to tissue-culture dishes. Clones were then screened for the expression of hBcl-2 by immunoblotting. Eight of 10 clones expressed the *hbcl-2* gene, irrespective of whether tet was added or omitted from the culture medium. Because this precluded us from comparing tet<sup>+</sup> with tet<sup>-</sup> cells, we used RAW-tTA cells to compare the rate of apoptosis with that of RAW-Bcl2 cells during DEP exposure.

#### Preparation of opsonized zymosan

Opsonized zymosan particles were prepared as previously described (38). Briefly, zymosan was incubated with normal mouse serum (1 mg/ml) at 37°C for 30 min, followed by two washes with HBSS. The opsonized zymosan particles were then resuspended in HBSS at a final concentration of 500 mg/ml.

#### Western blotting analysis for hBcl-2

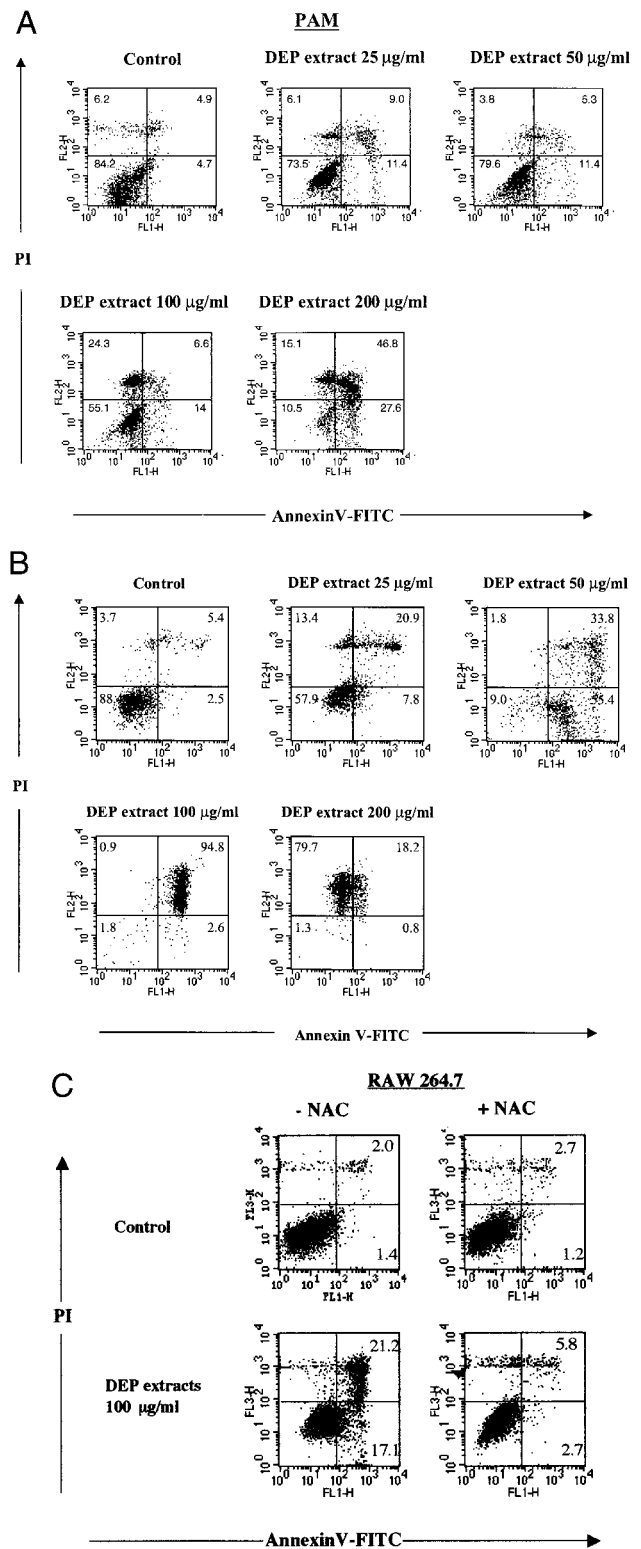
Cells ( $3 \times 10^6$ ) were grown in the presence and absence of 2  $\mu\text{g/ml}$  tet for 24 h. Cells were lysed as previously described, and 100  $\mu\text{g}$  lysate was separated by 12% SDS-PAGE and transferred to Immobilon-P membranes as described above. Immunoblotting for hBcl-2 was performed using a 1:1000 dilution of the anti-hBcl-2 Ab, followed by a 1:3000 dilution of HRP-coupled sheep anti-mouse Ab. To demonstrate that transfection with hBcl-2 increases total cellular Bcl-2 expression, we also performed Western blotting with a mAb that recognizes both the human and murine versions. This Ab was used at a 1:1000 dilution, followed by HRP-conjugated sheep anti-mouse secondary Ab at a dilution of 1:2000.

## Results

### Organic DEP extracts induce apoptosis in macrophages

We have recently shown that DEP phagocytosis leads to apoptosis in PAM and macrophage cell lines (19). In contrast, the carbonaceous core that remains after methanol extraction of the particle does not exert cytotoxic effects (19). This suggests that the methanol-extractable component is responsible for initiating PCD. We tested these methanol extracts in cellular apoptosis assays. First, the extracts were dried, resuspended in DMSO, and then added to rat PAM or the murine macrophage cell line RAW 264.7 for 18 h. Using dual annexin V/PI staining, we showed that the methanol-extractable components induce early (annexin V<sup>+</sup>/PI<sup>-</sup>) and late (annexin V<sup>+</sup>/PI<sup>+</sup>) apoptotic changes in primary as well as immortalized macrophages (Fig. 1). Although the toxic effects of the DEP extracts in both cell types were dose-dependent, there was a reproducible trend toward a lower death rate in PAM (Fig. 1A) compared with RAW 264.7 cells at every test concentration (Fig. 1B). This may reflect differences in metabolic processing of DEP chemicals in these cells, or differences in the abilities of these cells to defend themselves against oxidative stress.

DEP extracts generate ROS in lung microsomal preparations (7). Moreover, the acute toxic effects of DEP in mice exposed to the particles by intratracheal instillation can be reversed with superoxide dismutase (6), suggesting that oxidative stress plays a role in tissue injury by DEP chemicals. To investigate the association between ROS generation and RAW 264.7 cells, we first asked whether the antioxidant NAC could interfere with DEP-induced apoptosis. Prior incubation with 20 mM NAC interfered with apoptosis induction by 100  $\mu\text{g/ml}$  DEP extract (Fig. 1C). Although another synthetic SH compound, bucillamine, was also effective in inhibiting DEP extract-induced cell death at 20- to 40-fold lower concentrations than NAC, the flavanoids, silibinin and luteolin (39, 40), were ineffective in preventing cell death (Table I). Moreover, other antioxidants such as ascorbic acid (41) and PDTC (42) were also ineffective in inhibiting cellular toxicity (Table I). This suggests that the SH groups in NAC and bucillamine are responsible for the inhibitory effects of these antioxidants.



**FIGURE 1.** Organic DEP extracts induce apoptosis in macrophages. *A* and *B*, Two-color flow cytometry shows apoptosis in PAM and RAW 264.7 cells, respectively. Aliquots of  $3 \times 10^6$  cells were treated with the indicated concentrations of DEP extracts for 18 h. Cells were washed and stained with annexin V-FITC and PI as described in *Materials and Methods* and analyzed by two-color flow cytometry. The data in this experiment were reproduced once. The numbers in each quadrant reflect the percentage of cells in that population. *C*, Two-color flow cytometry demonstrated a decreased rate of apoptosis in RAW 264.7 cells treated with 100  $\mu\text{g/ml}$  of a DEP extract for 16 h in the presence of 20 mM NAC.

Table I. Comparison of the protective effects of different types of antioxidants

Drug	Dose	% PI-Positive Cells <sup>a</sup>
None <sup>b</sup>		71.9 ± 1.6
NAC	20 mM	16.9 ± 2.4
Bucillamine	0.1 mM	63.9 ± 1.1
	0.5 mM	17.9 ± 1.6
	1.0 mM	14.6 ± 1.6
PDTC	25 µg/ml <sup>c</sup>	99.8 ± 0.1
	50 µg/ml <sup>c</sup>	99.8 ± 0.0
	100 µg/ml <sup>c</sup>	99.8 ± 0.0
Ascorbic acid	25 µg/ml	97.3 ± 0.1
	50 µg/ml <sup>c</sup>	99.6 ± 0.1
	100 µg/ml <sup>c</sup>	99.7 ± 0.1
Silibinin	25 µg/ml	88.8 ± 1.2
	50 µg/ml	88.9 ± 0.2
	100 µg/ml	86.1 ± 1.0
Luteolin	25 µg/ml	90.2 ± 1.0
	50 µg/ml	90.6 ± 0.6
	100 µg/ml <sup>c</sup>	99.6 ± 0.0

<sup>a</sup> Percentage of PI-positive cells was determined by flow cytometry as described in *Materials and Methods*. Values represent the mean ± SEM.

<sup>b</sup> Cells were treated with 100 µg/ml DEP extract for 16 h.

<sup>c</sup> These agents induced significant cell death in the absence of the DEP extract.

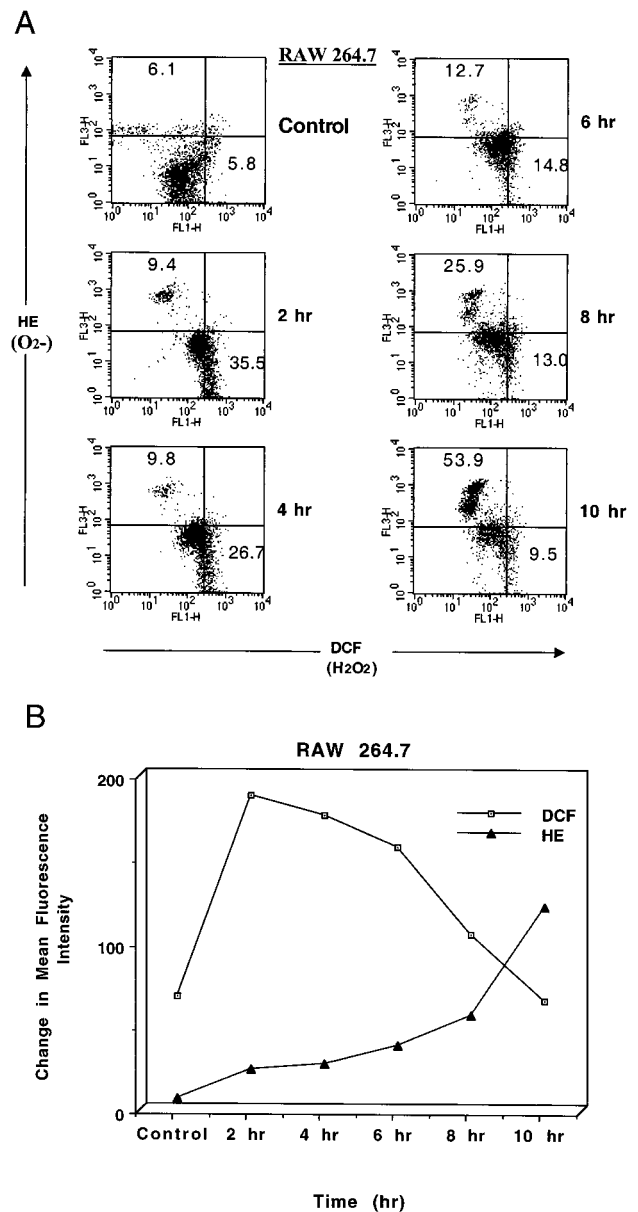
### Macrophages exposed to DEP extracts generate different types of ROS, and the pattern of ROS generation differs from that after zymosan ingestion

To determine the kinetics and types of ROS that are being produced by DEP chemicals, we used a two-color flow cytometry approach, which uses DCF-DA and HE (34). DCF-DA, which is freely cell permeable, is trapped in the cell after the acetate moieties are cleaved off and can be oxidized, mostly by H<sub>2</sub>O<sub>2</sub>, to a green fluorescent product. In contrast, HE is mostly oxidized by O<sub>2</sub><sup>-</sup> and forms ethidium bromide which emits red fluorescence (34). After introducing RAW 264.7 cells to the DEP extract, there was an immediate increase in DCF fluorescence, which peaked after 2 h and then returned to baseline over the next 6 h (Fig. 2A). For HE, there was a slow rise in the mean fluorescence intensity over the first 8 h, followed by a big rise in O<sub>2</sub><sup>-</sup> production from that point onwards (Fig. 2A). In a kinetic display of their mean fluorescence intensities, it can be seen that there are two independent phases of ROS production, namely an early phase of mostly H<sub>2</sub>O<sub>2</sub> production, followed by a later phase of O<sub>2</sub><sup>-</sup> production (Fig. 2B).

Macrophages also generate ROS through membrane-associated NADPH-oxidase during phagocytosis. To determine whether this respiratory burst activity leads to apoptosis, we performed two-color annexin V/PI and DCF fluorescence studies in RAW 264.7 cells during exposure to opsonized zymosan particles. Compared with cells exposed to DEP extract, the degree of apoptosis in zymosan-exposed RAW 264.7 cells was minimal (Fig. 3A). Although the DEP extract resulted in a sustained increase in DCF fluorescence over a 4-h observation period, zymosan-treated cells responded with H<sub>2</sub>O<sub>2</sub> production for 1 h only (Fig. 3B). Although this suggests that the magnitude or duration of ROS production may be a determinant for apoptosis, it is also possible that a specific chemical event may be involved in DEP-induced toxicity.

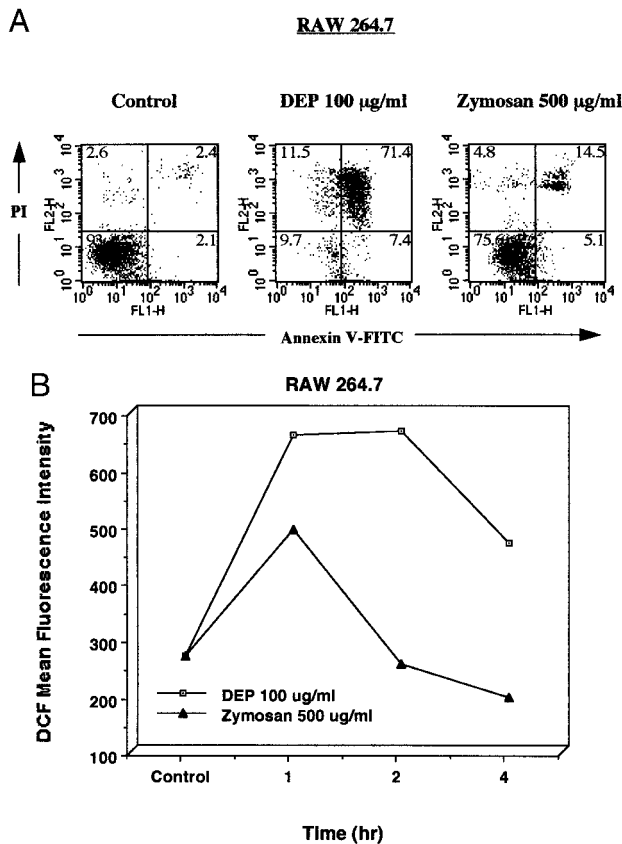
### DEP-induced apoptosis involves sequential mitochondrial dysfunction

The mitochondrial PT pore complex is regulated by numerous effectors, including divalent cations, protons, the thiol redox state, cross-linking of thiol groups, glutathione, ROS, lipid peroxidation, and function of the Bcl-2 complex (30–32). Moreover, redox-cycling chemicals and drugs also perturb mitochondrial function



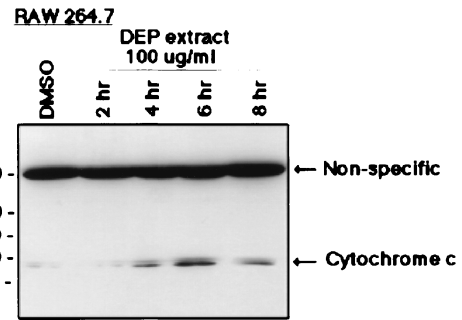
**FIGURE 2.** DEP extracts generate ROS, which plays a role in apoptosis. *A*, Two-color flow cytometry shows the effect of 100 µg/ml DEP extract on DCF fluorescence (H<sub>2</sub>O<sub>2</sub>) and HE conversion to ethidium (O<sub>2</sub><sup>-</sup>) in RAW 264.7 cells. The percentages of DCF- or HE-bright cells are indicated by the numbers in the lower right and upper left quadrants. Cellular staining and flow cytometry were conducted as described in *Materials and Methods*. *B*, Plotting of the increase in the mean fluorescence intensities for DCF and HE vs time. The data were obtained from the same flow cytometry experiment described in *A*. A second experiment yielded similar results.

(30). PT leads to the release of apoptogenic factors from the intermembrane space. Immunoblotting of a mitochondrial-free cytosolic fraction from RAW 264.7 cells showed the appearance of cytochrome *c* in the cytosol (Fig. 4). The kinetics of cytochrome *c* release, first seen around 4 h (Fig. 4), precedes the onset of annexin V staining, which commences between 4 and 6 h (19). Cytochrome *c* release in the cytosol leads to caspase-9 activation in the presence of Apaf-1 (25–28). This is compatible with our previous demonstration that DEP exposure leads to caspase-9 activation in macrophages (19).



**FIGURE 3.** Opsonized zymosan particles induce ROS but minimal apoptosis. *A*, Two-color flow cytometry shows apoptosis induced by opsonized zymosan. RAW 264.7 cells ( $3 \times 10^6$ ) were treated with opsonized zymosan (500  $\mu\text{g/ml}$ ) for 16 h. Staining of the cells with annexin V-FITC and PI and subsequent flow cytometric analysis were performed as described in Fig. 1. *B*, Flow cytometric analysis of DCF fluorescence ( $\text{H}_2\text{O}_2$ ) in RAW 264.7 cells. Cells were treated with 100  $\mu\text{g/ml}$  of DEP extract or 500  $\mu\text{g/ml}$  of opsonized zymosan for the indicated time periods. Staining of cells with DCF and PI was described in *Materials and Methods*. PI fluorescence was used to gate out the dead cells.

Opening of the PT pore allows the diffusion of solutes with a molecular mass of  $>1.5$  kDa (32). This results in an immediate dissipation of  $\Delta\psi_m$ , which can be assessed with the fluorochrome DiOC<sub>6</sub> (30). Assessment of DiOC<sub>6</sub> staining concurrent with HE labeling showed an orderly sequence of events in DEP extract-treated RAW 264.7 cells (Fig. 5A). First, there was a decrease in DiOC<sub>6</sub> staining, beginning at 2 h (Fig. 5A). This drop in  $\Delta\psi_m$  was progressive over the rest of the observation period (Fig. 5A). Another consequence of PT was the uncoupling of oxidative phosphorylation and interference in one-electron transfers in the mitochondrial inner membrane (32). This led to  $\text{O}_2^-$  production which was detected by HE conversion (32, 43). In DEP extract-treated cells, a prominent phase of  $\text{O}_2^-$  production started around 8 h, leading to the generation of DiOC<sub>6</sub><sup>low</sup>/HE<sup>high</sup> and DiOC<sub>6</sub><sup>low</sup>/HE<sup>low</sup> populations (Fig. 5A). The kinetics of  $\text{O}_2^-$  production in this experiment are compatible with the kinetics of  $\text{O}_2^-$  production, shown in Fig. 2B. Although DEP extracts have similar effects on  $\Delta\psi_m$  and  $\text{O}_2^-$  production in PAM, the magnitude of these effects in PAM was not as prominent as in RAW 264.7 cells (Fig. 5B). In PAM, the DEP extract induced a single population of DiOC<sub>6</sub><sup>low</sup> cells, some of which (26%) showed an increase in  $\text{O}_2^-$  production (Fig. 5B). This is consistent with the lesser toxicity of DEP extracts in rat PAM (Fig. 1B).



**FIGURE 4.** Western blot analysis showing cytochrome *c* release in the cytosol of RAW 264.7 cells treated with 100  $\mu\text{g/ml}$  of a DEP extract for the indicated time periods. Aliquots of  $3 \times 10^6$  RAW 264.7 cells were incubated with 100  $\mu\text{g/ml}$  DEP extract for the indicated time period. Cells were lysed in a hypotonic buffer, and the S-100 fraction was collected and subjected to immunoblotting as described in *Materials and Methods*. The experiment was reproduced once.

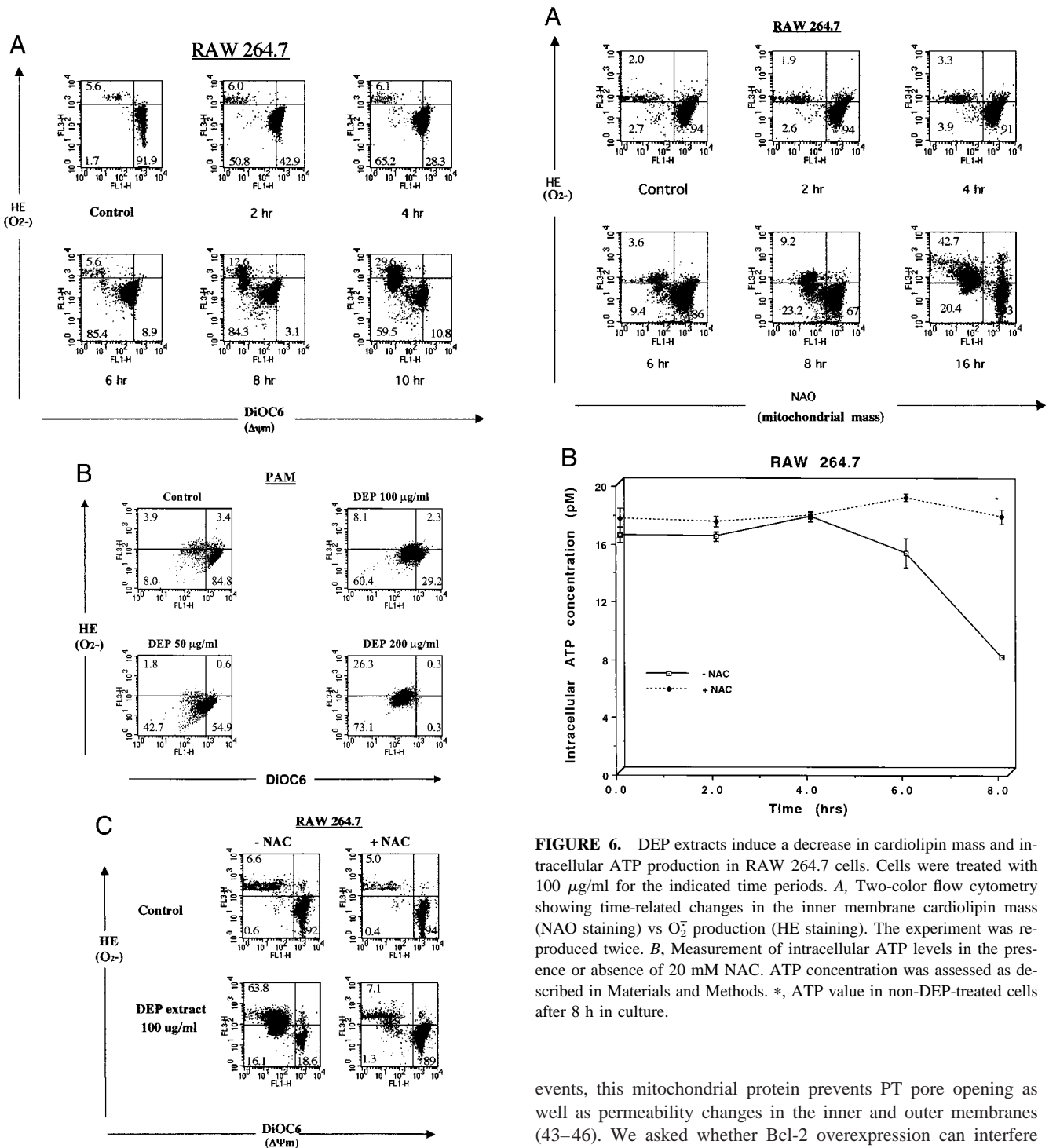
To demonstrate that the dissipation of  $\Delta\psi_m$  is linked to chemical-induced oxidative stress or thiol cross-linking effects, we studied the effect of NAC on DiOC<sub>6</sub>/HE fluorescence. Our data demonstrate that NAC effectively blocked the  $\Delta\psi_m$  decrease as well as the increase in  $\text{O}_2^-$  generation in RAW 264.7 cells (Fig. 5C). NAC had similar protective effects in PAM (not shown). Although a redox-cycling quinone, menadione, also induced a decrease in  $\Delta\psi_m$ , which could be inhibited by NAC, opsonized zymosan particles had no demonstrable effect on  $\Delta\psi_m$  (not shown). Among the possible explanations for the difference between the chemicals and zymosan is that respiratory burst activity does not perturb mitochondrial function, while chemical-induced oxidative stress does. Another possibility is that redox-cycling chemicals induce PT by direct complexing to pore proteins. In this regard, it is interesting that NAC also protects mitochondria against dexamethasone-induced cell death (32).

To study the effect of DEP chemicals on the structural integrity of the mitochondrial inner membrane, we stained macrophages with a fluorochrome, NAO, which binds stoichiometrically to an inner membrane phospholipid, cardiolipin (35). After the introduction of a DEP extract to RAW 264.7 cells, NAO fluorescence intensity decreased precipitously from  $\sim 6$  h onward (Fig. 6A). Moreover, the concurrent measurement of  $\text{O}_2^-$  production (HE conversion), showed the characteristic rise in ROS production from 8 h onward (Fig. 6A). Menadione had similar effects (not shown). Similar observations were made in rat PAM, except that the magnitude of the decrease in NAO staining and number of  $\text{O}_2^-$ -producing cells in the case of PAM were lower than in RAW 264.7 cells (not shown).

Massive induction of PT with cumulative damage to the mitochondrial inner membrane can uncouple oxidative phosphorylation, leading to interference in ATP synthesis (26, 27). An assessment of cellular ATP levels showed a steep drop in this nucleotide pool from  $\sim 6$  h onward (Fig. 6B). Moreover, previous incubation of RAW 264.7 cells with NAC prevented the decline in ATP levels (Fig. 6B), showing that the impact of DEP chemicals on the mitochondrion is either through ROS generation or a direct effect on the PT pore.

#### *Bcl-2* overexpression causes a slight delay but does not protect against chemical-induced apoptosis

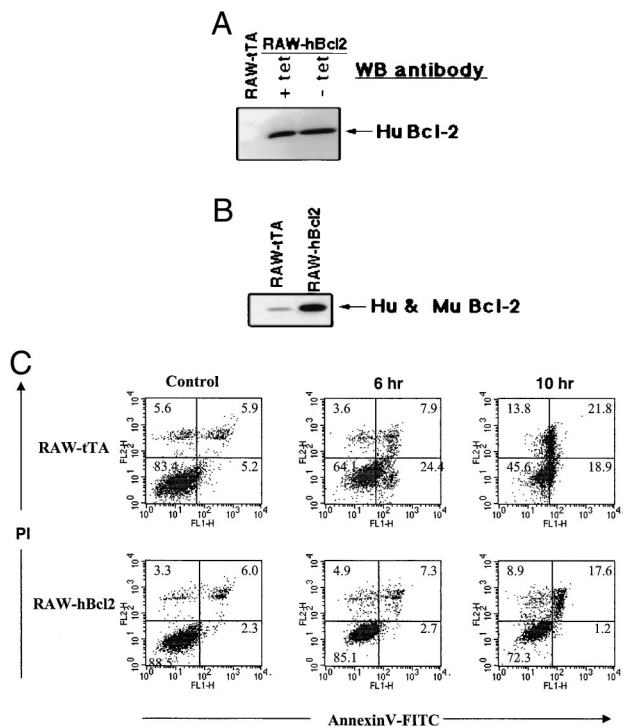
PT constitutes the first rate-limiting event of a common pathway of apoptosis and can be induced either in a Bcl-2-regulated or in a Bcl-2-independent fashion (32). In the case of Bcl-2-regulated



**FIGURE 5.** DEP extracts induce a decrease in  $\Delta\psi_m$  as well as an increase in  $O_2^-$  production in a NAC-dependent manner. **A**, Aliquots of  $3 \times 10^6$  RAW 264.7 cells were treated with 100  $\mu\text{g}/\text{ml}$  of the DEP extract for the indicated time periods before dual staining with HE and DiOC<sub>6</sub> as described in Materials and Methods. Two-color flow cytometry demonstrated the development of a DiOC<sub>6</sub><sup>low</sup>/HE<sup>low</sup> population at 2–6 h. This develops into a DiOC<sub>6</sub><sup>low</sup>/HE<sup>high</sup> population beyond 8 h. **B**, Rat PAM were treated with 50–100  $\mu\text{g}/\text{ml}$  DEP extracts for 10 h before assessment of two-color HE vs DiOC<sub>6</sub> fluorescence. In contrast to RAW 264.7, this cell type yields a single DiOC<sub>6</sub><sup>low</sup> population, in which some (26%) of the cells show increased  $O_2^-$  production. This analysis was reproduced once. **C**, Effect of 20 mM NAC on the DiOC<sub>6</sub>/HE profile in RAW 264.7 cells treated with 50 or 100  $\mu\text{g}/\text{ml}$  DEP extracts for 10 h.

**FIGURE 6.** DEP extracts induce a decrease in cardiolipin mass and intracellular ATP production in RAW 264.7 cells. Cells were treated with 100  $\mu\text{g}/\text{ml}$  for the indicated time periods. **A**, Two-color flow cytometry showing time-related changes in the inner membrane cardiolipin mass (NAO staining) vs  $O_2^-$  production (HE staining). The experiment was reproduced twice. **B**, Measurement of intracellular ATP levels in the presence or absence of 20 mM NAC. ATP concentration was assessed as described in Materials and Methods. \*, ATP value in non-DEP-treated cells after 8 h in culture.

events, this mitochondrial protein prevents PT pore opening as well as permeability changes in the inner and outer membranes (43–46). We asked whether Bcl-2 overexpression can interfere with the proapoptotic effects of DEP chemicals in RAW 264.7 cells. This was accomplished by stable transfection of RAW-tTA cells with a *hBcl-2* construct using the tet-regulated vector, pUHD10–3 (for details see *Materials and Methods*; Ref. 36). Although tet was not effective in suppressing Bcl-2 expression in stably selected RAW-hBcl2 cells (Fig. 7A), we were able to compare these cells with RAW-tTA cells that contain a lesser amount of endogenous Bcl-2 (Fig. 7B). Addition of a DEP extract to RAW-tTA cells induced apoptotic (annexin V<sup>+</sup>/PI<sup>-</sup>) changes in 24% of the cells after 6 h (Fig. 7C). In contrast, extract-treated hBcl-2 overexpressing cells showed only 2.7% annexin V-positive cells at this point (Fig. 7C). However, by 10 h there was an advanced degree of apoptosis in hBcl-2-overexpressing cells, indicating that DEP-induced toxicity is largely a Bcl-2-independent



**FIGURE 7.** Effect of hBcl-2 overexpression on DEP-induced apoptosis in RAW 264.7 cells. RAW-tTA cells, which stably express the tet-regulated transactivator protein (tTA), were transfected with a pUHD10-3-hBcl2 and pTRE-hygro vectors as described in *Materials and Methods*. Cells were stably selected in hygromycin for 3 wk. Individual colonies were picked and grown in tissue culture dishes. *A*, Western blot analysis shows hBcl-2 expression in a representative clone (of eight). All of the selected clones expressed hBcl-2 irrespective of the presence of absence of tet. The reason for this lack of tet regulation is unexplained as transfection of RAW-tTA cells with the pUHD13.3 vector (tet-O operon linked to luciferase) did show reporter gene activity that could be suppressed by tet. *B*, Western blot analysis to compare total Bcl-2 expression in RAW-tTA vs RAW-hBcl2 as judged by a different antiserum that detects both human and murine Bcl-2. *C*, Two-color flow cytometry showing the effect of a 100  $\mu$ g/ml DEP extract on apoptosis in RAW-tTA vs RAW-hBcl2 cells in the absence of tet.

process. In accordance with these findings, Bcl-2 overexpression did not prevent a decrease in  $\Delta\psi_m$  or cardiolipin degradation in extract-treated cells (not shown).

## Discussion

In this paper, we show that organic DEP extracts induce apoptosis in macrophages, including PAM. Although synthetic SH antioxidants prevented cell death, other classes of antioxidants did not interfere with cellular toxicity. The apoptotic event was associated with ROS production and the effects on mitochondrial function before and at the onset of apoptosis. Mitochondrial perturbation leads to an orderly sequence of events, which commences with a decrease in  $\Delta\psi_m$ , followed by cytochrome *c* release and development of membrane asymmetry (annexin V labeling). Structural damage to the mitochondrial inner membrane, as evidenced by a decreased cardiolipin mass, leads to a disruption in one-electron transfer,  $O_2^-$  generation, and uncoupling of oxidative phosphorylation. NAC prevented these mitochondrial changes, suggesting that ROS are involved in the mitochondrial process or that NAC may complex to the chemicals and/or the PT pore to prevent this damage. Overexpression of the hbc1-2 oncogene delayed but did not prevent apoptosis.

DEP are composed of a carbon core containing unburnt and adsorbed petrochemicals (21). Methanol extracts made from DEP induce ROS production in lung microsomes in a cytochrome P450 reductase-dependent manner (12). Although several lung cell types may contribute to radical production, our results show that PAM are able to increase  $H_2O_2$  and  $O_2^-$  production in response to exposure to organic DEP extracts (Fig. 1). Uncoupling of electron transfer in the mitochondrion is a constant feature accompanying apoptosis in a variety of different cell types (32, 43). This likely is the source for the delayed  $O_2^-$  production shown in Fig. 2. Although the source of  $H_2O_2$  production is uncertain, this may be from a microsomal localized enzyme complex involved in the metabolism or transformation of DEP chemicals (12). It is also interesting that  $H_2O_2$  production by zymosan-induced respiratory burst activity was associated with only a small rise in cell death (Fig. 3). Although this observation may have several explanations, two possibilities are that the magnitude and duration of ROS production differ between these stimuli or that the ROS produced by membrane-associated NADPH-oxidase do not impact the mitochondrion.

Among the >400 chemicals that are present in DEP, PAH, nitro-derivatives of PAH, and oxygenated PAH derivatives (ketones, quinones, diones) are candidate chemicals that may contribute to ROS generation (8–11). In this regard, Kumagai et al. (12) have shown that the oxidative effects of DEP extracts can be neutralized by  $NaBH_4$ , arguing that quinones participate in ROS production in vitro. Quinones are reduced to semiquinone radicals by microsomal localized cytochrome P450 reductases (47). These semiquinone radicals produce ROS and can be reoxidized to the original quinone group, thereby initiating a futile redox cycle (47, 48). Therefore, it is interesting that menadione, a naphthoquinone that is readily reduced via NADPH-cytochrome P450 reductase to a semiquinone radical (49), had similar effects on the mitochondrion and cellular apoptosis as DEP chemicals (not shown). PAH may contribute to further ROS production during cytochrome P4501A1-dependent transformations. Characterization of the actual chemical groups responsible for ROS generation in macrophages needs to await the fractionation of DEP chemicals into functional groups. In addition to organic chemical compounds, the contribution of metal ions, such as  $Fe^{2+}$ , should also be considered as a source of ROS generation (50).

ROS production by redox-cycling quinones or PAH may be involved in DEP-induced apoptosis. ROS can induce lipid peroxidation or cross-linking of thiol groups in proteins, both of which can trigger PT (29, 32). Therefore, not surprisingly, mitochondria have been implicated in the induction of apoptosis by a growing list of pro-oxidative chemicals, including redox-cycling quinones and PAH (29, 51–53). Although the data showing that NAC and buccillamine interfere with DEP-induced apoptosis are compatible with the involvement of oxidative stress (Fig. 1C and Table I), there are other possible explanations for the role of these synthetic SH compounds. One is that the reduced thiol groups in these antioxidants may directly couple to quinones and the PT pore, thereby preventing chemical damage to this pore. Because this covalent interaction may also prevent the redox cycling of quinones, the concomitant decrease in ROS production may appear to correlate with a decreased rate of cell death. Failure of other classes of antioxidants to protect against DEP-induced apoptosis favors the latter notion (Table I).

Once perturbed by oxidative stress or covalently coupled DEP chemicals, the PT pore initiates a sequence of events leading to apoptosis. The first is a fall in  $\Delta\psi_m$ , detectable by reduced labeling with DiOC<sub>6</sub> (Fig. 5). This leads to or is associated with the release



of apoptogenic factors, including cytochrome *c* (Fig. 4). Cytochrome *c* complexes with Apaf-1, inducing it to associate with procaspase-9 and triggering caspase-9 activation (27). This initiator caspase triggers a further proteolytic cascade that ultimately induces cell death. In this regard, we have previously demonstrated that DEP-induced apoptosis is associated with procaspase-9 cleavage (19). Once apoptosis is underway, mitochondria undergo further damages, including structural damage to the inner membrane (Fig. 6A). This leads to or is associated with defects in one-electron transfers and is likely responsible for  $O_2^-$  production (Fig. 2A, 5, A and B, and 6A). At this stage, macrophages still have a normal size and normal dye exclusion (no PI labeling), but may exhibit asymmetric phosphatidylserine distribution in the membrane (detected by annexin V staining). This stage is followed by cell shrinkage, apoptotic blebbing, and increased membrane permeability (PI staining).

Intact mitochondria produce ATP. When PT involves a large fraction of mitochondria in a cell, ATP may become depleted because of uncoupled oxidative phosphorylation (Fig. 5B). This event may be responsible for our finding that caspase inhibitors are only partially effective in interfering with DEP-induced apoptosis (19). If ATP depletion becomes severe, apoptosis may transition into necrosis. This combined apoptosis-necrosis event is seen at higher DEP extract concentrations (e.g., >100  $\mu\text{g}/\text{ml}$  in RAW 264.7 cells). Necrosis may augment the pulmonary effects of DEP because necrotic cell fragments induce further inflammation.

PT constitutes the first rate-limiting event of the common pathway of apoptosis and can be induced either in a Bcl-2-regulated or in Bcl-2-independent fashion (32, 43). One way to distinguish between those possibilities is through Bcl-2 overexpression, which should interfere with the former but not the latter event. Although overexpression of hBcl-2 somewhat retarded the rate of apoptosis (Fig. 7B), this was not effective in ultimately decreasing the overall rate of cell death (Fig. 7C). Moreover, hBcl-2 overexpression did not prevent the extract-induced decrease in  $\Delta\psi_m$  or NAO fluorescence in extract-treated cells (not shown). This suggests that DEP-induced apoptosis is Bcl-2 independent.

Apoptosis may play an important role in the pulmonary toxicity of DEP. First, widespread apoptosis in PAM may interfere with lung defense against infectious organisms such as viruses. In this regard, it has been demonstrated that previous DEP exposure leads to increased mortality in mice infected with the influenza virus (54). DEP pre-exposure also interferes with the clearance of indicator particles from the airways of mice challenged via intratracheal instillation (55). Second, apoptosis of PAM may spread DEP chemicals via apoptotic bodies to neighboring tissue cells, thereby increasing tissue damage. In this way, phagocytosed apoptotic bodies may serve as a type of Trojan horse. Third, the mechanism of apoptosis in macrophages may also apply to neutrophils and other respiratory cell types. For instance, bronchial epithelial cells contribute to PM-induced respiratory inflammation and ROS production (56). Epithelial shedding, putatively as a consequence of apoptosis, may contribute to bronchial hyperreactivity in people with asthma. Fourth, macrophage apoptosis is associated with ROS production. Because many of these radicals are released into the extracellular environment, they may impact neighboring cells, which lead to their activation or apoptosis. Finally, an understanding of DEP toxicity will help to understand the toxicity of other inhalable particulates. An in vitro model of macrophage apoptosis may prove useful in characterizing the chemical compounds in other types of PM that induce acute respiratory effects.

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