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## Induction of Heme Oxygenase-1 Expression in Macrophages by Diesel Exhaust Particle Chemicals and Quinones via the Antioxidant-Responsive Element

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# Induction of Heme Oxygenase-1 Expression in Macrophages by Diesel Exhaust Particle Chemicals and Quinones via the Antioxidant-Responsive Element<sup>1</sup>

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Diesel exhaust particles (DEP) contain organic chemicals that contribute to the adverse health effects of inhaled particulate matter. Because DEP induce oxidative stress in the lung and in macrophages, effective antioxidant defenses are required. One type of defense is through the expression of the antioxidant enzyme, heme oxygenase I (HO-1). HO-1 as well as phase II detoxifying enzymes are induced via antioxidant response elements (ARE) in their promoters of that gene. We show that a crude DEP total extract, aromatic and polar DEP fractions, a benzo(a)pyrene quinone, and a phenolic antioxidant induce HO-1 expression in RAW264.7 cells in an ARE-dependent manner. *N*-acetyl cysteine and the flavonoid, luteolin, inhibited HO-1 protein expression. We also demonstrate that the same stimuli induce HO-1 mRNA expression in parallel with the activation of the SX2 enhancer of that gene. Mutation of the ARE core, but not the overlapping AP-1 binding sequence, disrupted SX2 activation. Finally, we show that biological agents, such as oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, could also induce HO-1 expression via an ARE-dependent mechanism. Prior induction of HO-1 expression, using cobalt-protoporphyrin, protected RAW264.7 cells against DEP-induced toxicity. Taken together, these data show that HO-1 plays an important role in cytoprotection against redox-active DEP chemicals, including quinones. *The Journal of Immunology*, 2000, 165: 3393–3401.

We are interested in the adverse effects of organic chemical compounds from diesel exhaust particles (DEP)<sup>3</sup> on the lung and the immune system (1). One example is the exacerbation of allergic inflammation in the lung due to the adjuvant effects of DEP on IgE production (1–4). We and others have shown that macrophages are important cellular targets for DEP in the lung, and that chemical extracts made from DEP induce reactive oxygen species (ROS) in these cells (5–8). The induction of oxidative stress leads to macrophage activation (e.g., cytokine production) or to cytotoxic effects such as apoptosis and necrosis (6–8). Guarding against these oxidative cellular effects are antioxidant defense pathways that seek to restore the cellular redox

equilibrium to normal limits. In this communication we will explore the role of a transcriptional regulatory element, the antioxidant response element (ARE) or electrophile-responsive element, as a defense strategy against oxidant chemicals in DEP extracts (9–17).

The ARE, with underlined consensus sequence GTGAC-NNNGC, is a transcriptional regulatory element that plays a role in the expression of phase II enzymes such as glutathione-*S*-transferase, NAD(P)H:quinone oxidoreductase, and glucuronosyltransferase (9–14). The ability of these enzymes to conjugate redox-cycling chemicals is an important protective mechanism against electrophile and oxidative toxicity. Prototypical chemicals are phenolic antioxidants, Michael reaction acceptors, isothiocyanates, trivalent arsenicals, and redox-cycling polycyclic aromatic hydrocarbons (PAH) and quinones (10–17). Although this antioxidant defense mechanism has been studied extensively as a hepatic detoxification mechanism, it has also been suggested that the ARE pathway may contribute to antioxidant defenses in the lung (18). An example is regulation of heme oxygenase-1 (HO-1) expression by the SX2 enhancer, a 268-bp gene fragment that is located 4 kb upstream of the start site (18–20). This enhancer contains two AREs and can be activated by some of the chemical species listed above (20). Because DEP contain a variety of organic substances that undergo redox cycling (21, 22), we were interested in whether DEP extracts may induce HO-1 expression (23). HO-1 confers protection against oxidative lung injury (24, 25) and may therefore play a role in the pulmonary defense against particulate pollutants. We were particularly interested in the role of oxygenated PAH such as quinones in this pathway, because these chemicals have been implicated in ROS generation by DEP extracts in lung microsomal preparations (24, 25).

HO-1 protein expression can be induced throughout the lung, with particular abundance in the alveolar and bronchiolar epithelium as well as airway inflammatory cells, including macrophages

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<sup>3</sup> Abbreviations used in this paper: DEP, diesel exhaust particles; ARE, antioxidant response element; BPQ, benzo(a)pyrene quinone; CoPP, cobalt protoporphyrin; DCF, dichlorofluorescein diacetate; GC/MS, gas chromatography/mass spectrometry; HO-1, heme oxygenase-1; Luc, luciferase; NAC, *N*-acetyl-cysteine; OxPAPC, oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PAH, polycyclic aromatic hydrocarbon; PI, propidium iodide; PM, particulate matter; ROS, reactive oxygen species; SIM, single ion monitoring; tBHQ, *tert*-butylhydroquinone.

(24, 25). Although bronchoalveolar macrophages have an important role in DEP clearance from the airway (5), macrophage activation by DEP may contribute to the pulmonary inflammation and enhanced IgE production attributed to this particulate matter (PM) (1, 26). Because the ARE pathway is operational in macrophages, it is possible that this pathway may play a role in the defense against DEP chemicals, including PAH and quinones (27). In this regard we have previously shown that *b*-naphthoflavone (a PAH) and *tert*-butylhydroquinone (tBHQ; a quinone and a phenolic antioxidant) induce ARE activation in macrophages (27).

We wished to determine whether DEP chemicals, including PAH and quinone fractions, induce antioxidant defenses through an effect on HO-1 expression in macrophages. We demonstrate that DEP total extracts, polar and aromatic DEP fractions, as well as benzo(a)pyrene-3,6-quinone (BPQ) induce HO-1 expression in parallel with transcriptional activation of AREs in the HO-1 enhancer. These events could be suppressed by *N*-acetyl-cysteine (NAC) or mutagenesis of the AREs in the SX2 enhancer. This establishes a biologically relevant role for the ARE in antioxidant defenses in macrophages.

## Materials and Methods

### Reagents

DMEM, macrophage serum-free medium, penicillin-streptomycin, L-glutamine, and FBS were purchased from Life Technologies (Gaithersburg, MD). DEP were a gift from Dr. Masaru Sagai (National Institute of Environmental Studies, Tsukuba, Ibaraki, Japan). Oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (OxPAPC) was a gift from Dr. Judith Berliner (University of California, Los Angeles, CA). NAC, tBHQ, propidium iodide (PI), silibinin, and LPS were obtained from Sigma (St. Louis, MO). Anti-HO-1 mAb was purchased from Stressgen (Victoria, Canada). Sheep anti-mouse Ab was obtained from Amersham (Arlington Heights, IL). A luciferase assay kit was purchased from PharMingen (San Diego, CA). Enhanced chemiluminescence reagents were obtained from Pierce (Rockford, IL). An endotoxin-free Maxiprep kit was purchased from Promega (Madison, WI). TRIzol RNA extraction reagent, reverse transcriptase, and Taq DNA polymerase were obtained from Life Technologies. Dichlorofluorescein diacetate (DCF) was purchased from Molecular Probes (Eugene, OR). Luteolin was obtained from Calbiochem (La Jolla, CA). Cobalt protoporphyrin (CoPP) was purchased from Porphyrin Products (Logan, UT).

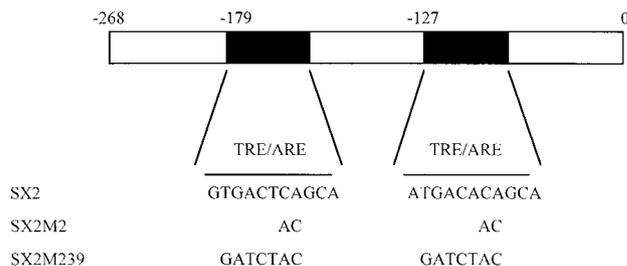
### Cell culture and transfection

RAW264.7 were cultured as previously described (6). Plasmid DNA was prepared using the endotoxin-free Maxiprep kit according to the manufacturer's instructions (Promega). The sequences of the wild-type and mutant SX2 elements (SX2, M2, and M239) in the 5'-upstream region of the mouse HO-1 gene have been described previously (18–20) (Fig. 1). These enhancer elements were subcloned into the pD44-luciferase plasmid vector. Transfections were conducted using previously described methods (27). Briefly,  $7 \times 10^6$  RAW264.7 cells resuspended in DMEM containing 20% FBS were transfected with 20  $\mu$ g of the reporter cDNA using an electroporator (Bio-Rad, Hercules, CA) with voltage set at 260 V and capacitance set at 975  $\mu$ F. Cells were resuspended in DMEM containing 10% FBS and were rested for 24 h before the addition of various stimuli as shown in the figure legends.

### Cellular stimulation and inhibition with antioxidants

The tBHQ, BPQ, silibinin, and luteolin stock solutions were prepared in DMSO. DEP extracts were prepared as previously described (6). Briefly, 100 mg DEP was suspended in 25 ml of methanol and sonicated for 30 s. The DEP/methanol suspension was centrifuged at 2000 rpm for 10 min at 4°C. The methanol supernatant was transferred to a preweighed polypropylene tube and dried under nitrogen gas (6). Dried DEP extracts were resuspended in DMSO at 100 mg/ml and stored at -20°C in the dark. The NAC stock solution (1 M) was made in HEPES immediately before use and was diluted to a final concentration of 20 mM in culture medium. The antioxidants were added 2 h before and during cellular stimulation. CoPP stock (25  $\mu$ g/ $\mu$ l) was made in 0.1 M NaOH and stored at -80°C. For HO-1 induction, CoPP was diluted in the culture medium to the desired concentrations, after which the pH was adjusted, and the medium was sterile-filtered. An equal amount of NaOH was added to the control medium.

### The SX2 enhancer



**FIGURE 1.** Diagram of the SX2 enhancer of the HO-1 gene. This enhancer contains two antioxidant response elements at positions -179 and -127 (numbering from the 3' end of the enhancer) with overlapping tetra- and pentanucleotide response elements (TRE or AP-1 binding sites) (20). The wild-type enhancer as well as mutations thereof were inserted into the pD44-luciferase reporter. The SX2-M2 mutant contains base pair changes in the AP-1 site, leaving the ARE core sequence intact. The SX2-M239 mutant contains base pair changes that affect the ARE core sequence.

OxPAPC in chloroform (2 mg/ml) was stored at -80°C, dried under nitrogen gas, and resuspended in culture medium immediately before use. RAW264.7 cells were plated at  $10^6$  cells/well in 3 ml of culture medium for 24 h before the addition of various stimuli. Controls were treated with DMSO at a final concentration of 0.1%.

### Western blotting to determine HO-1 expression

The cells were harvested by scraping and were lysed as previously described (6). Fifty micrograms of total lysate protein was electrophoresed on SDS-polyacrylamide gels before transfer to nitrocellulose membranes. Blots were sequentially overlaid with anti-HO-1 mAb at 0.3 mg/ml and sheep anti-mouse Ab conjugated to peroxidase according to the manufacturer's instruction. Blots were developed with the enhanced chemiluminescence reagent according to the manufacturer's instructions.

### RT-PCR analysis

Total RNA was extracted using TRIzol RNA extraction reagent (1). RT was conducted at 42°C for 1 h in a total volume of 20  $\mu$ l containing 5  $\mu$ g of total RNA; 0.5 mg of oligo(dT)<sub>12-18</sub>; 10 mM DTT; 0.5 mM each of dATP, dGTP, dCTP, and dTTP; and 10 U of Superscript II reverse transcriptase. HO-1 primers for PCR amplification of a 668-bp fragment from exon 1 of mouse HO-1 gene (28) were obtained from Life Technologies. The forward (sense) primer was 5'-CTGTGTAACCTCTGCTGTCC-3', and the reverse (antisense) primer was 5'-CCACACTACCTGAGTCTACC-3'. Primers for mouse  $\beta$ -actin mRNA were used as an internal control. The forward (sense) primer was 5'-TGGAAATCCTGTGGCATC ATGAAAC-3', and the reverse (antisense) primer was 5'-TAAAACG CAGCTCAGTAACAGTCCG-3'. PCR was performed in a total volume of 25  $\mu$ l, using 4  $\mu$ l of cDNA template, 0.5 mM of the sense and antisense primers, 2.5 U of Taq DNA polymerase, and 2 mM MgCl<sub>2</sub> in a Perkin-Elmer thermocycler (Norwalk, CT). After amplification for 35 cycles (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C), PCR products were electrophoresed in 2% agarose gels and viewed by ethidium bromide staining.

### Luciferase (Luc) assay

Transfected cells were collected by scraping and were lysed with the luciferase lysis buffer according to the manufacturer's instruction. Luc assays were performed on 50 mg lysate protein, using the manufacturer's assay kit and a Monolith 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA).

### DEP fractionation and gas chromatography/mass spectrometry (GC/MS) analysis

One gram of DEP was extracted with 60 ml of methylene chloride five times using a Vir-Tis homogenizer (Virtis, Gardiner, NY). The combined extracts were concentrated by rotoevaporation, and asphaltenes were precipitated by exchange into hexane. The supernatant was concentrated, dried

over anhydrous sodium sulfate, and subjected to silica gel column chromatography (column size, 1 × 30 cm) following the method of Venkatesan et al. (29). Aliphatic, aromatic, and polar fractions were collected by elution with 20 ml of hexane, 40 ml of hexane/methylene chloride (3/2), and 30 ml of methylene chloride/methanol (1/1), respectively. The fractions were weighed in a microbalance by evaporating off a known volume of an aliquot of the sample made up in methylene chloride or methanol.

GC with Flame ionization detector (Varian 3400 GC with a SPI injector and a DB-5 column, 30 m, 0.25 mm id, 0.25 μm film; Varian, Walnut Creek, CA) was used to chemically characterize the alkane fraction. The aromatic and polar fractions were analyzed by a gas chromatograph/mass spectrometer (Finnigan 4000 (Finnigan-MAT, San Jose, CA) equipped with a Varian 3400 GC with a split/splitless injector and Galaxy 2000 for Windows data system; Los Gatos Circuits, Los Gatos, CA). The PAHs were quantitated by GC/MS in the selective ion monitoring (SIM) mode using deuterated internal standard mixtures, and an electron energy of 70 eV was used. The mass spectrometer was operated on SIM mode, using appropriate optimum windows to include the quantitation and confirmation masses for the analytes. A five-point response factor calibration curve was established. Standard concentrations used to construct the calibration curve were 1, 5, 10, 20, and 50 ng/μl. The identification of compounds detected at concentration above the method detection limit (MDL) was double checked by the confirmation ions. If the concentration of the target analyte exceeded the linear range of the calibration standards, the fraction was either concentrated or diluted and reanalyzed. The polar fraction was taken up in dichloromethane/acetonitrile (1/1, v/v) and analyzed by GC-MS in the SIM mode and the SCAN mode to search for oxy-PAHs, quinones, and ketones using total ion count spectra and the National Institute of Standards and Technology library.

#### Flow cytometric analysis

ROS generation was determined by DCF fluorescence, and cell viability was determined by PI uptake as previously described (6). Briefly, cells were incubated in 2.5 μM DCF in DMEM (10<sup>6</sup>/ml) at 37°C in the dark for 30 min. PI (2.5 μg/ml) was added before the analysis. For viability analysis to study the effect of CoPP, cells were stained with PI (2.5 μg/ml) for 15 min at room temperature in the dark. Flow cytometry was performed using a FACScan equipped with an argon laser (Becton Dickinson, Franklin Lakes, NJ). The mean fluorescence intensities for DCF and PI were analyzed in FL-1 and FL-2 channels, respectively.

#### Statistics

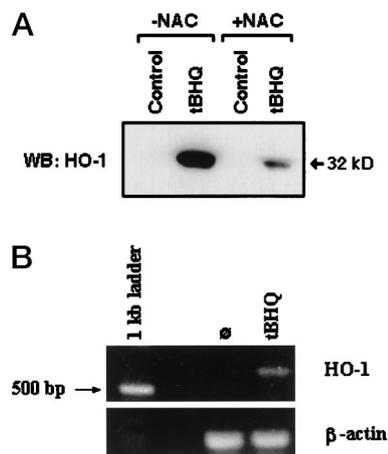
Data were analyzed using SAS statistical software (SAS Institute, Cary, NC). Scheffe's method of multiple comparisons with F test was used for ANOVA.

## Results

### Tert-BHQ induced HO-1 expression in RAW264.7 cells

A variety of stimuli can induce HO-1 expression both in vitro and in vivo. These factors include bacterial endotoxin (LPS), IL-1, hyperoxia, heavy metals, as well as some electrophilic phase 2 enzyme inducers (18, 30, 31). Moreover, Prestera et al. reported that a prototypic phase II enzyme inducer, tBHQ, induces HO-1 gene expression in mouse hepatoma cells (20). We tested the same chemical in RAW264.7 cells. Following incubation with tBHQ for 16 h, RAW264.7 cells showed HO-1 expression, as determined by immunoblotting (Fig. 2A). Inclusion of NAC in the culture medium significantly reduced HO-1 expression (Fig. 2A). Treatment of RAW264.7 cells with tBHQ also induced HO-1 mRNA expression, as determined by RT-PCR analysis (Fig. 2B).

In addition to its role in heme catabolism, HO-1 plays an important role as a stress protein with cytoprotective effects (32, 33). A common feature of nonheme HO-1 inducers, such as tBHQ, is their stimulatory effect on the SX2 enhancer in that gene (20). This gene fragment contains two consensus ARE sites at positions -179 and -127 (numbering from the 3' end of the SX2 domain; Fig. 1) (20). We transfected RAW264.7 cells with the SX2 fragment linked to a Luc reporter. Subsequent treatment with tBHQ induced a 4-fold increase in SX2-Luc activity (Fig. 3A). For protein expression, this response was significantly suppressed by 20 mM NAC (Fig. 2A).



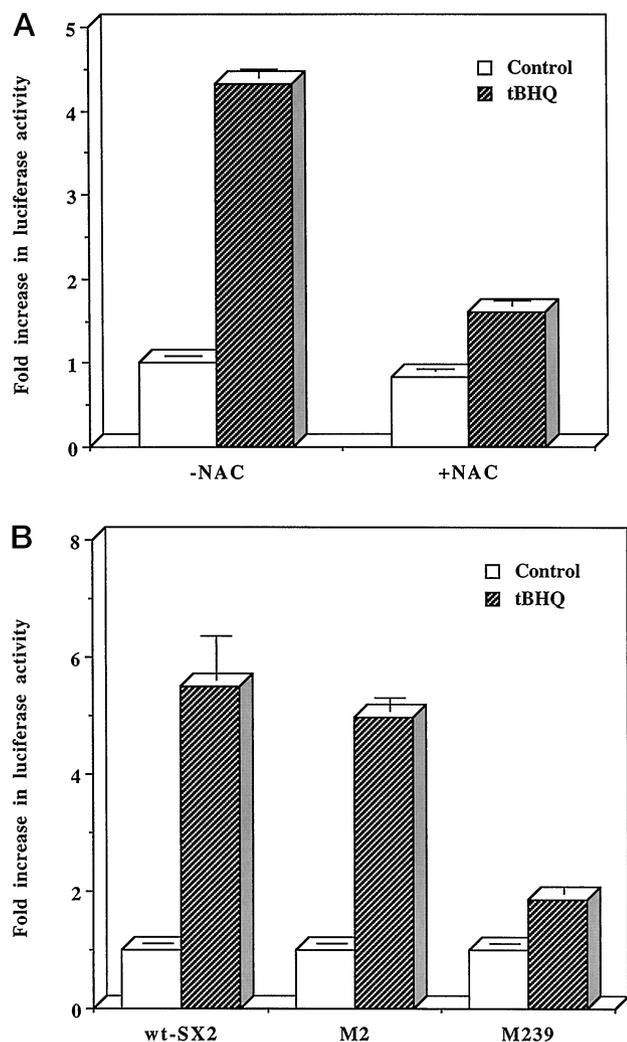
**FIGURE 2.** Western blot and RT-PCR analysis to show induction of HO-1 expression by tBHQ. *A*, Western blotting of RAW264.7 lysates made from cells treated with 50 mM tBHQ for 16 h in the presence and the absence of 20 mM NAC. The blotting membrane was overlaid sequentially with 0.3 μg/ml anti-HO-1 followed by a 1/2000 dilution of the secondary sheep anti-mouse peroxidase-labeled Ab. *B*, RT-PCR analysis of HO-1 mRNA in RAW264.7 cells treated with 50 μM tBHQ for 6 h. RT-PCR analysis was performed as described in *Materials and Methods*.

To demonstrate that AREs play an important role in the activation of the SX2 enhancer, we also studied the effect of tBHQ on a mutant reporter (SX2-M239-Luc), in which ARE core sequences have been changed (Fig. 1) (18–20). Our results demonstrate a significant reduction in the SX2-M239-Luc response to tBHQ treatment compared with the wild-type reporter (Fig. 3B). Because these AREs contain an overlapping AP-1 nucleotide sequence (GTGACTCAGC) that putatively plays a role in regulating transcriptional activity, we compared the effect of tBHQ on the SX2-M2-Luc mutant that eliminates the AP-1 binding site but leaves the ARE core sequence (GTGACTCAGC) intact (Fig. 1). This mutation did not interfere with the transcriptional activation of the SX2 enhancer (Fig. 3B).

### Induction of HO-1 expression and promoter activity by organic DEP extracts is sensitive to the inhibitory effects of NAC and flavonoid antioxidants

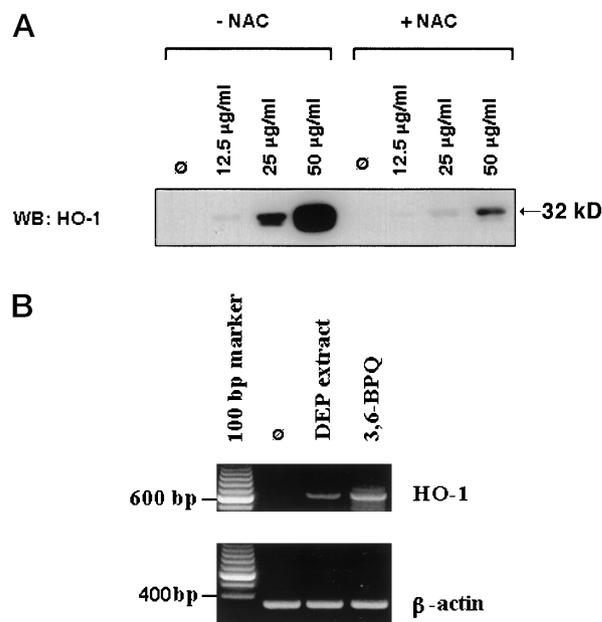
Because DEP contain a wide variety of chemicals that may induce oxidative stress (6), including quinones and ketones (22, 23), we asked whether a methanol extract made from these particles could induce HO-1 expression in RAW264.7 cells. RAW264.7 cells were incubated with these extracts after they were dried and re-suspended in DMSO (6). Western blot analysis showed a dose-dependent increase in HO-1 expression (Fig. 4A), while RT-PCR analysis showed induction of HO-1 mRNA expression by the DEP extract (Fig. 4B).

NAC suppressed HO-1 expression by DEP chemicals (Fig. 4A). This effect may be explained by the role of NAC as an antioxidant or its ability to directly complex to chemical groups such as quinones (34). Because a DEP extract is a known inducer of ROS that can be suppressed by NAC in macrophages (6), we asked whether other classes of antioxidants interfere with HO-1 expression. For that purpose, we employed two flavonoid antioxidants, silibinin and luteolin (35, 36). Although silibinin had minimal inhibitory effects on enhanced DCF fluorescence, luteolin led to substantive inhibition of DEP-induced ROS generation at all concentrations tested (Fig. 5A). When used in an assay for HO-1 induction, luteolin, but not silibinin, interfered with HO-1 expression in a dose-dependent fashion (Fig. 5B). Taken together, these data show that oxidative stress is involved in DEP-induced HO-1 expression.



**FIGURE 3.** Reporter gene assays showing effect of tBHQ on SX2 enhancer activity. RAW264.7 cells transiently transfected with 20  $\mu\text{g}$  of either the wild-type or mutant SX2 reporters were rested for 24 h before treatment with 50  $\mu\text{M}$  tBHQ for 16 h in the presence or the absence of 20 mM NAC. The controls were treated with an equal amount of DMSO. *A*, Representative experiment showing the effect of NAC on wild-type SX2 reporter activity (mean  $\pm$  SEM). The stimulatory effect of tBHQ ( $p < 0.001$ ) and the inhibitory effect of NAC ( $p < 0.001$ ) were statistically significant in four different experiments. Values are the mean  $\pm$  SEM ( $n = 4$ ;  $p < 0.001$ ). *B*, The effects of SX2 mutation on the induction of reporter activity by tBHQ. Wild-type SX2 is compared with the AP-1 mutant (SX2-M2) as well as a mutant that disrupts the ARE core sequence (SX2-M239). Mutations in the ARE core sequences resulted in a significant reduction ( $p < 0.001$ ) in the SX2-Luc activity compared with the wild-type promoter. The experiment was repeated three times. Values are the mean  $\pm$  SEM.

In the next set of experiments, we looked at the effects of DEP extracts on the activation of a transfected SX2-Luc reporter. This showed SX2-Luc activity with similar dose-dependence as shown in the protein expression assay (Fig. 6A). Thus, while a dose of 12.5  $\mu\text{g}/\text{ml}$  had a slight effect, doses of 25 and 50  $\mu\text{g}/\text{ml}$  induced progressively greater responses. These concentrations are below the DEP extract doses leading to apoptosis, i.e., concentrations  $>50$   $\mu\text{g}/\text{ml}$  (6). Prior addition of NAC significantly reduced SX2 activation (Fig. 6A). Mutation of the core AREs (i.e., using SX2-M239-Luc), but not the overlapping AP-1 site, resulted in a significant decrease in reporter gene activity (Fig. 6B). Taken together, these results show that DEP chemicals play a role in HO-1



**FIGURE 4.** Induction of HO-1 expression in RAW264.7 cells by DEP extracts. Cells were stimulated with the indicated concentrations of DEP extracts for 16 h in the presence or the absence of 20 mM NAC. *A*, Western blot showing dose-dependent induction of HO-1 protein expression by DEP extracts. Immunoblotting was performed as described in Fig. 3A. *B*, RT-PCR analysis showing HO-1 mRNA expression in RAW264.7 cells treated with 50  $\mu\text{g}/\text{ml}$  DEP or 20  $\mu\text{M}$  3,6-BPQ for 6 h. RT-PCR was performed as described in *Materials and Methods*. Mouse  $\beta$ -actin was used as internal control.

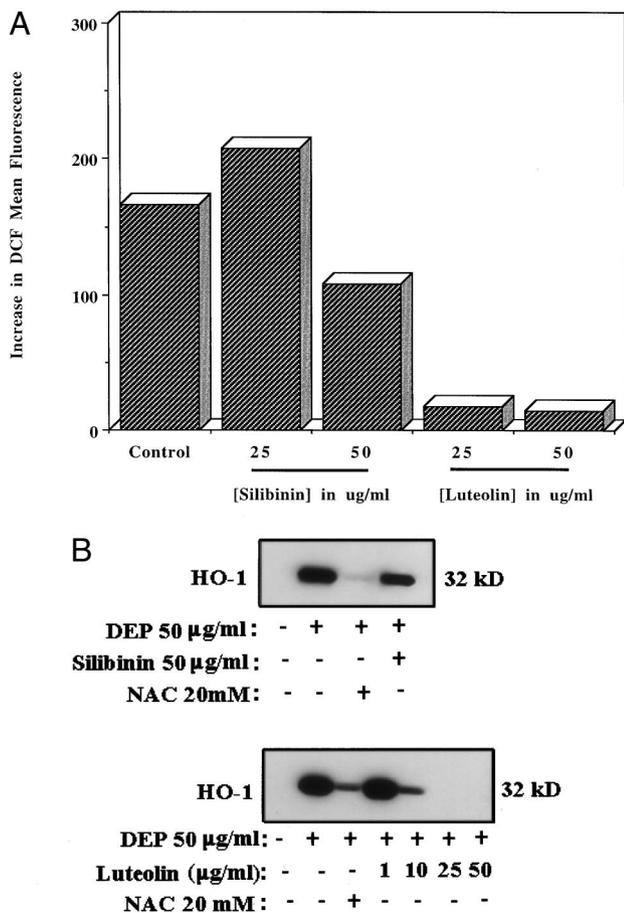
expression via stimulation of AREs in the SX2 domain of that gene.

#### *Aromatic and quinone-enriched DEP fractions induce HO-1 expression*

Previous studies of the effect of extractable diesel chemicals on ROS generation in the lung have suggested that redox cycling quinones are responsible for that effect (22, 23). Because this result was based on interference of  $\text{NaBH}_4$  in the activity of a crude methanol extract, we separated a methylene chloride extract obtained from DEP into three fractions using silica gel chromatography (Table I). The relative abundance of aliphatic, aromatic, and polar components is shown in Table I. The GC analysis of aliphatic fraction showed the presence of mainly normal alkanes, ranging in carbon number from 13 to 31 with  $\text{C}_{17}$  to  $\text{C}_{24}$  homologues predominating (not shown). The PAHs, which were present in the aromatic fraction, were characterized and quantitated by GC/MS and are listed in Table II. Although the polar fraction did not contain any measurable PAHs, oxygenated compounds, such as 9-fluorene-9-one, anthracene/phenanthrenol, and 9,10-anthracenedione, were identified in this fraction (Table III). Using the aromatic and polar fractions, we were able to elicit dose-dependent HO-1 expression in RAW264.7 cells (Fig. 7). The polar fraction was more potent than the aromatic fractions and induced a response at 1  $\mu\text{g}/\text{ml}$  (Fig. 7). This is considerably less than the amount of crude DEP extract required for response induction, i.e., 25  $\mu\text{g}/\text{ml}$  or more (Fig. 4A). The aliphatic fractions had no discernible effect on HO-1 expression (Fig. 7).

#### *Differential effects of benzo(a)pyrene quinones on HO-1 induction*

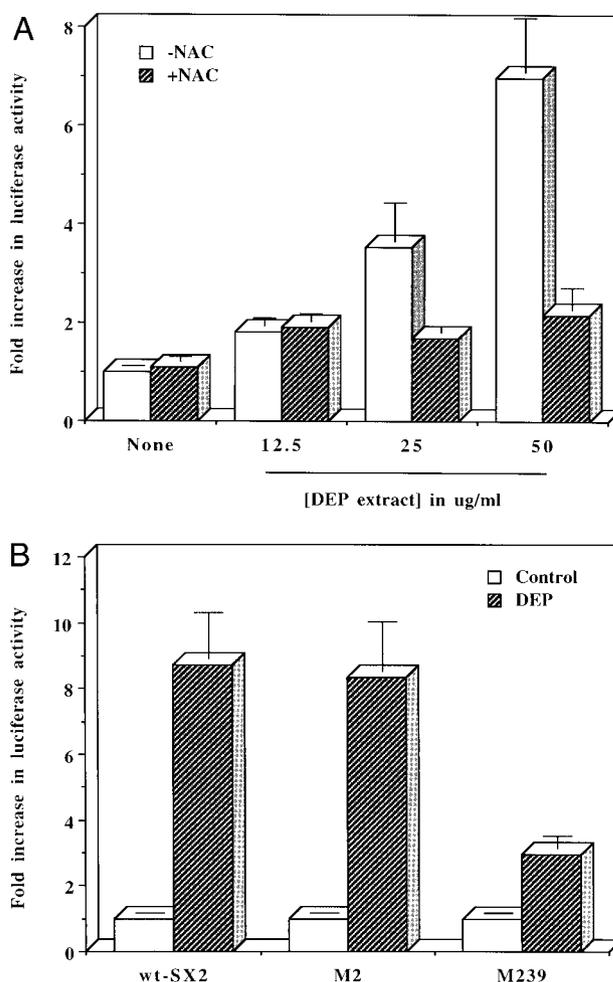
The increased potency of the polar fraction on HO-1 expression suggests that oxygenated PAHs such as quinones are involved. To



**FIGURE 5.** The effects of flavonoid antioxidants on cellular ROS and HO-1 expression. *A*, Flow cytometric analysis of cellular ROS. Cells were pretreated with silibinin or luteolin at the indicated concentrations for 2 h. The cells were then incubated with 100  $\mu$ g/ml crude DEP extract in DMEM in the presence of the antioxidants. The amount of cellular ROS was determined by DCF/PI double staining and flow cytometry. PI was used to gate out the dead cells. The increase in DCF fluorescence was calculated by subtracting the mean fluorescence intensity of extract-treated cells from that of untreated cells. *B*, Western blot demonstrating the effects of silibinin (*upper panel*) and luteolin (*lower panel*) on HO-1 expression. RAW264.7 cells pretreated with silibinin or luteolin for 2 h were stimulated with 50  $\mu$ g/ml crude DEP extract for 5 h in the continuous presence of antioxidants. NAC (20 mM) was used in parallel as a positive control.

further explore the role of quinones, we looked at the effect of a frequently studied quinone that is present in second-hand smoke and in PM, albeit in minor amounts (23, 37). Oxidation of the BaP ring yields three species, namely 1,6-, 3,6-, and 6,12-BPQ (23). We asked whether these quinones participate in HO-1 expression in RAW264.7 cells. Exposure to 20  $\mu$ M of each of these quinones showed that while 3,6-BPQ was an effective inducer of HO-1 protein expression, the 1,6- and 6,12-quinones failed to do so (Fig. 8A). 3,6-BPQ also induced HO-1 mRNA expression, as determined by RT-PCR analysis (Fig. 4B). HO-1 induction by this quinone was completely abolished by 20 mM NAC (Fig. 8A). Using the SX2-Luc promoter, we could elicit a small (2.5-fold), but statistically significant, increase in luciferase activity with the 3,6-BPQ, but not the 1,6- or 6,12-BPQ, species (not shown).

The comparatively low level of stimulation of 3,6-BPQ may be due to quinone reactivity with proteins, e.g., the FBS in the culture medium. For that reason we asked whether conducting the experiment in serum-free medium will lower the concentration at which



**FIGURE 6.** The effect of DEP extract on wild-type and mutant SX2 enhancer activity. *A*, Reporter gene assay to show the effect of NAC on the wild-type SX2 enhancer. Cells were treated with the indicated amounts of the DEP extracts ( $\mu$ g/ml) in the presence or the absence of 20 mM NAC for 16 h. Values are the mean  $\pm$  SEM ( $n = 4$ ;  $p < 0.01$ ). *B*, Reporter assay to show the effect of the AP-1 (*M2*) and ARE core (*M239*) mutants on the activation of the SX2 enhancer by DEP extracts. Cellular transfection and luciferase activity were performed as described in *Materials and Methods*. The response reduction in the SX2-M239-Luc reporter to 50  $\mu$ g/ml extract was statistically significant ( $p < 0.001$ ). Values are the mean  $\pm$  SEM.

HO-1 is induced by 3,6-BPQ. We compared the effect of 3,6-BPQ on HO-1 expression at 2 and 20  $\mu$ M in the presence or the absence of 10% FBS in RAW264.7 cells. Our data show that in the absence of FBS, HO-1 expression could be obtained at both quinone concentrations, while in the presence of 10% FBS, only the 20- $\mu$ M dose was stimulatory (Fig. 8B). Note that the basal level of HO-1 expression is increased under serum-free conditions. This may be due to the depletion of antioxidants from the culture medium, or it may reflect cellular stress of serum depletion.

#### Evidence that HO-1 has cytoprotective effects in RAW264.7 cells

There is abundant evidence that HO-1 is a stress protein with cytoprotective effects, including pulmonary defense against LPS and hyperoxia. To determine whether HO-1 was able to protect RAW264.7 against the cytotoxic effects of DEP extracts, these cells were previously incubated with CoPP, an inducer of HO-1 activity. CoPP has been shown to exert cytoprotective effects *in vivo* in a rat liver transplantation model (38). Western blotting showed that CoPP induced HO-1 expression at a dose  $\geq 7.5$   $\mu$ g/ml

Table I. Gravimetric distribution of fractions from methylene chloride extract of DEP (g/g of DEP)<sup>a</sup>

Aliphatic	Aromatic (PAH)	Polar
0.24	~0.07 <sup>b</sup>	0.16

<sup>a</sup> About 64% (by weight) of DEP was methylene chloride extractable. One gram DEP contained 0.166 g asphaltene.

<sup>b</sup> Not weighed. Measured by difference only.

(Fig. 9A). The lower effective concentration was used to treat RAW264.7 cells for 48 h before treatment with 100 µg/ml of a crude DEP extract. We have previously shown that this dose induces apoptosis in an oxidative stress-dependent fashion (6). Using flow cytometric analysis to detect PI uptake in dead cells, we showed that the DEP extract induced cell death in >90% RAW264.7 cells (Fig. 9B). NAC interfered with this event (22% dead cells; Fig. 9B). In cells pretreated with CoPP, the rate of cell death fell to 54% (Fig. 9B), suggesting that under conditions of HO-1 expression, cells are protected against the toxicity of DEP chemicals.

#### Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (PAPC) induce HO-1 expression

In addition to chemical agents, it has been demonstrated that some biological agents, such as bacterial LPS, could induce human ARE reporter activity and HO-1 expression in RAW264.7 cells (18, 31). This effect may be relevant to pulmonary protection against inhaled bacterial endotoxin as well as during Gram-negative sepsis (39). Another biological agent that induces HO-1 expression in endothelial cells and smooth muscle cells and that has relevance to macrophages is oxidized LDL (40). Oxidized LDL as well as one of the phospholipids contained therein, PAPC, has stimulatory effects on macrophages (35, 36). We asked, therefore, whether OxPAPC could induce HO-1 expression in RAW264.7 cells. OxPAPC could induce HO-1 expression as determined by immunoblotting (Fig. 10A). RT-PCR analysis demonstrated that RAW264.7 stimulation with OxPAPC increased HO-1 mRNA expression (Fig. 10B). This response was suppressed by 20 mM NAC (Fig. 10A). Using the SX2-Luc reporter, we could demonstrate that OxPAPC induced robust enhancer activity in an NAC-sensitive manner (Fig. 10C). Taken together, these data closely resemble the effect of oxidant

chemicals on HO-1 expression in RAW264.7 cells. OxPAPC should therefore be included in the growing list of biological agents that impact the HO-1 antioxidant defense pathway.

## Discussion

In this paper we demonstrate that a variety of chemical agents as well as the oxidized phospholipid, OxPAPC, induce HO-1 expression in macrophages. The effects of tBHQ; 3,6-BPQ; crude DEP extracts; aliphatic, aromatic, and polar DEP fractions; and OxPAPC on HO-1 expression could be abrogated by NAC pretreatment. In addition, we demonstrated that the flavonoid, luteolin, also interfered with the ability of DEP extracts to induce HO-1 expression. The above stimuli also activate the SX2 enhancer of the HO-1 gene. Similar to its effect on protein expression, NAC interfered with SX2 activation, suggesting that oxidative stress is involved in the transcriptional activation of this gene. This response requires electrophile or antioxidant responsive elements, suggesting that HO-1 participates in cellular protection against oxidative stress in a manner analogous to phase II enzyme induction. It is relevant, therefore, that induction of HO-1 expression by CoPP protected against the cytotoxic effects of DEP extract.

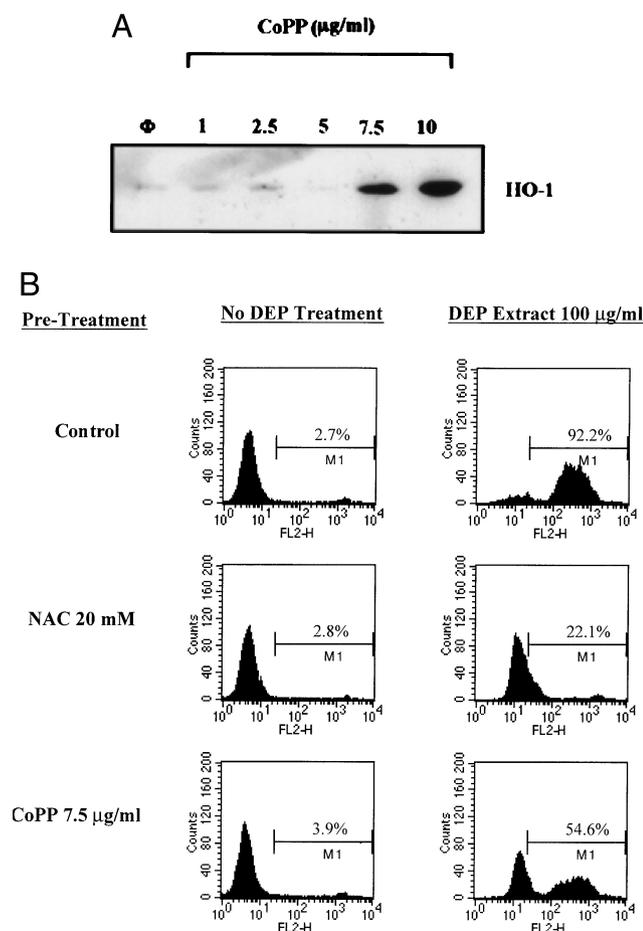
The ARE is a transcriptional regulatory element that is a widely recognized protector against chemical-induced electrophile and oxidative toxicity (9–17). This is accomplished through the expression of phase II conjugating enzymes, e.g., NADPH:quinone oxidoreductase. This enzyme performs two-electron reductions on quinones and converts them to hydroxyl quinones, which are no longer able to participate in one-electron reductions or redox-cycling events (41). Phase II enzymes therefore effectively remove the source of the electrophile and ROS, which are also the primary inducers of ARE activity. This induction of ARE activity involves activation or intracellular translocation of a constitutively expressed transcription factor in hepatocytes and macrophages (11, 27). Recently, it has been shown that members of the Nrf transcription factor family interact with the ARE (42–44), and that Nrf2 regulates induction of the HO-1 gene (45). On exposure to electrophilic agents, the DNA-binding activity of Nrf2 is markedly induced, while the Nrf2 steady state mRNA levels remain constant (45, 46). This suggests that signals from oxidizing agents are transduced from a cellular sensor to Nrf proteins. Nrf2 contains a negative regulatory domain that interacts with a novel cytoplasmic protein, Keap1 (46). Keap1 is liberated from Nrf2 in the presence

Table II. GC/MS analysis of PAH in the aromatic fraction from DEP<sup>a</sup>

PAH	Quantity (µg/g DEP)	PAH	Quantity (µg/g DEP)
Naphthalene	0.4	Pyrene	8.2
2-Methylnaphthalene	0.9	Benz(a)anthracene	3.9
1-Methylnaphthalene	0.8	Chrysene/triphenylene	12.0
2,6-Dimethylnaphthalene	2.0	Benzo(k)fluoranthene	8.9
2,3,5-Trimethylnaphthalene	2.5	Benzo(b)fluoranthene	6.0
Biphenyl	4.6	Benzo(e)pyrene	7.8
Acenaphthylene	<0.4	Benzo(a)pyrene	<0.5
Acenaphthene	<0.4	Anthracene	6.0
Fluorene	1.4	9,10-Diphenylanthracene	<0.5
2-Methylfluorene	19.0	Perylene	<0.5
Phenanthrene	64.6	Ideno(1,2,3-cd)pyrene	<0.5
3,6-Dimethylphenanthrene	19.2	Dibenz(a,h)anthracene	<0.5
1-Methylphenanthrene	47.2	Picene	<0.5
2,3-Benzofluorene	1.8	Benzo(ghi)perylene	<0.5
1,1'-Binaphthalene	<0.4	Anthanthrene	<0.5
Dibenzothiophene	<1.3	Coronene	<0.5
Fluoranthene	17.8	1,2,4,5-Dibenzopyrene	<0.5

<sup>a</sup> The amount of PAH in the aromatic fraction of DEP extract was quantitated as described in *Materials and Methods*.

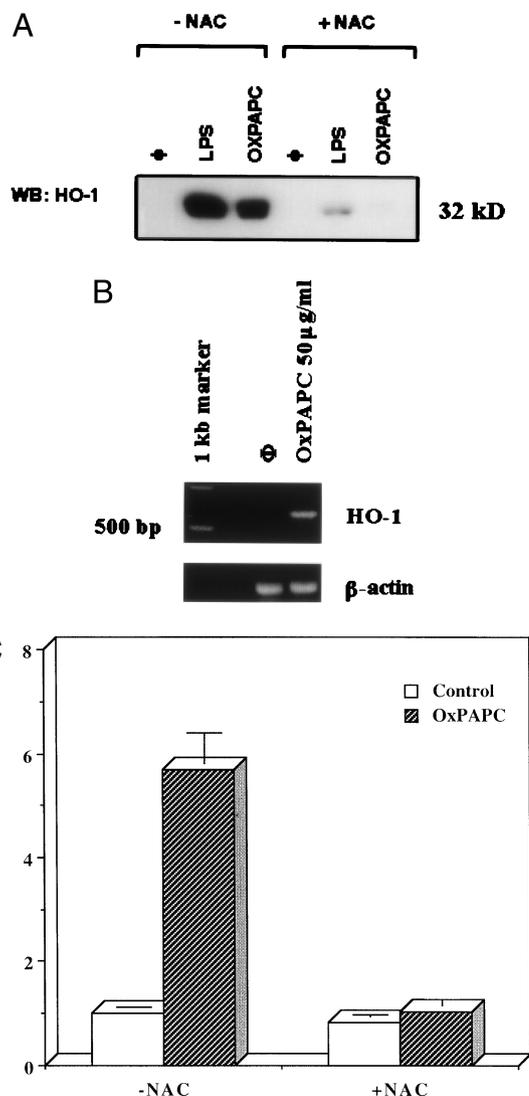




**FIGURE 9.** CoPP has a cytoprotective effect in RAW264.7 cells. **A**, Dose-dependent induction of HO-1 by CoPP. RAW264.7 cells were treated with CoPP for 48 h at the indicated concentrations. Western blotting was performed as described in *Materials and Methods*. **B**, Flow cytometric analysis showing that pretreatment with 7.5 µg/ml CoPP for 48 h protected against DEP-induced cellular toxicity. Cells were treated with 100 µg/ml of a DEP extract for 16 h. NAC (2-h pretreatment) was used as a positive control. Cell viability was determined by PI staining (2.5 µg/ml;  $10^6$  cells). *M1*, Percentage of PI-positive cells.

be explained both through its antioxidative effects as well as by its directly complexing to quinones, it is relevant that luteolin also suppressed ROS production and HO-1 expression (Fig. 5). That strengthens the idea that oxidative stress is involved in this event. Interestingly, another flavonoid, silibinin, was ineffective in suppressing HO-1 induction (Fig. 5B). This may be related to the fact that this agent is a good scavenger for hypochlorous acid (HOCl), but not of  $O_2^-$  (51). Taken together, we propose that oxygenated PAH and quinones are strong candidates responsible for the observed HO-1 expression induced by the DEP extracts.

The role of the ARE in macrophages is not confined to protection against chemical effects alone. In this regard, mildly oxidized LDL and a phospholipid component of LDL, OxPAPC, also induced HO-1 expression in RAW264.7 cells (40). Moreover, it has been reported that HO-1 is induced in vascular endothelial and smooth muscle cells by oxidized LDL in parallel with other stress proteins (40, 52). These results suggest that HO-1 induction by OxPAPC may have a protective effect against arterial wall inflammation, such as that seen in atherosclerosis. This protective effect may also apply to other forms of inflammation where IL-1 and TNF- $\alpha$  come into play, because it has been demonstrated that these



**FIGURE 10.** The effects of OxPAPC on HO-1 expression in RAW264.7 cells. **A**, Western blotting showing HO-1 expression by OxPAPC. RAW264.7 cells were stimulated with 50 µg/ml OxPAPC for 16 h in the presence or the absence of 20 mM NAC. Immunoblotting was performed as described in Fig. 3A. **B**, RT-PCR analysis showing HO-1 mRNA expression in RAW264.7 cells treated with 50 µg/ml OxPAPC for 6 h. Mouse  $\beta$ -actin was used as an internal control. **C**, Reporter assay to show the effect of NAC on SX2 enhancer activity in RAW264.7 cells. The cells were transiently transfected with the wild-type SX2 promoter-reporter and stimulated with 50 µg/ml OxPAPC for 16 h. OxPAPC induced a statistically significant increase ( $p < 0.001$ ) in wild-type SX2-Luc activity, which was suppressed by 20 mM NAC. Values are the mean  $\pm$  SEM ( $n = 4$ ).

cytokines induce HO-1 expression (53, 54). The ARE may therefore have biological functions beyond the detoxification of chemicals.

In summary, we show that DEP chemicals and quinones induce an antioxidant defense pathway in macrophages through an ROS-mediated effect on the ARE. Because this effect appears to be induced at lower chemical concentrations than those causing cytotoxic effects, the ARE pathway could theoretically be exploited to enhance antioxidant defenses against PM.

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