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Nrf2 Is a Key Transcription Factor That Regulates Antioxidant Defense in Macrophages and Epithelial Cells: Protecting against the Proinflammatory and Oxidizing Effects of Diesel Exhaust Chemicals[1]

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The proinflammatory effects of particulate pollutants, including diesel exhaust particles (DEP), are related to their content of redox cycling chemicals and their ability to generate oxidative stress in the respiratory tract. An antioxidant defense pathway, which involves phase II enzyme expression, protects against the pro-oxidative and proinflammatory effects of DEP. The expression of enzymes, including heme oxygenase-1 (HO-1) and GST, is dependent on the activity of a genetic antioxidant response element in their promoters. In this study we investigated the mechanism by which redox cycling organic chemicals, prepared from DEP, induce phase II enzyme expression as a protective response. We demonstrate that aromatic and polar DEP fractions, which are enriched in polycyclic aromatic hydrocarbons and quinones, respectively, induce the expression of HO-1, GST, and other phase II enzymes in macrophages and epithelial cells. We show that HO-1 expression is mediated through accumulation of the bZIP transcription factor, Nrf2, in the nucleus, and that *Nrf2* gene targeting significantly weakens this response. Nrf2 accumulation and subsequent activation of the antioxidant response element is regulated by the proteasomal degradation of Nrf2. This pathway is sensitive to pro-oxidative and electrophilic DEP chemicals and is also activated by ambient ultrafine particles. We propose that Nrf2-mediated phase II enzyme expression protects against the proinflammatory effects of particulate pollutants in the setting of allergic inflammation and asthma. ***The Journal of Immunology*****, 2004, 173: 3467–3481.**

There is increasing evidence that particulate pollutants exert adverse pulmonary effects by generating airway inflammation as well as acting as adjuvants for IgE production in the immune system (1–5). These actions could explain increased asthma prevalence and airway hyper-reactivity in response to elevated levels of ambient particulate matter (PM)[3] (1–5). However, fundamental uncertainty and disagreement still exist about what physical and chemical properties determine PM health risks, what pathophysiological mechanisms are operative, and what air quality regulations should be adopted to deal with these health risks (6). Particle size and composition are two key variables that determine health impact (6–8). In this regard, we have demonstrated that ultrafine PM (size range, <0.15 μm) exert more toxicity in target cells than particles with larger aerodynamic diameter, i.e., fine (0.1–2.5 μm) and coarse (2.5–10 μm) PM (7). The increased toxicity of ultrafines can be explained by their small size, large airborne number concentration, high organic carbon (OC) content, high content of polycyclic aromatic hydrocarbons (PAHs), and ability to localize in subcellular organelles, such as mitochondria (7, 8). Moreover, ultrafine particles gain access to the systemic circulation and may be involved in inflammatory events in the vessel wall, e.g., atherosclerosis (9).

To explain the proinflammatory effects of PM, a link has been established with their ability to generate reactive oxygen species (ROS) (1, 10, 11). This includes superoxide radical ($O_2^{\cdot-}$) production in macrophages, bronchial epithelial cells, and lung microsomes incubated with diesel exhaust particles (DEP) or their organic extracts (12–18). Moreover, we have demonstrated that thiol antioxidants, which suppress ROS production, interfere in the adjuvant effects of DEP in vivo (19). How do ROS lead to airway inflammation? When ROS production exceeds the cell's ability to neutralize or inactivate these oxygen radicals, a state of oxidative stress ensues (20, 21). Oxidative stress activates a number of redox-sensitive signaling pathways, including several MAPK cascades (1, 22, 23). The MAPKs play a role in the expression of proinflammatory cytokines, chemokines, and adhesion molecules (1). In the course of studying these redox signaling pathways, we observed that MAPK activation and cytokine production require

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[3] Abbreviations used in this paper: PM, particulate matter; ARE antioxidant response element; CHX, cyclohexamide; DAPI, 4′,6-diamido-2-phenylindole hydrochloride; DEP, diesel exhaust particle; EC, elemental carbon; HO-1, heme oxygenase-1; NAC, *N*-acetylcysteine; Nrf2, NF-E2-related factor-2; NQO1, NAD(P)H-quinone oxidoreductase 1; $O_2^{\cdot-}$, superoxide radical; OC, organic carbon; PAH, polycyclic aromatic hydrocarbon; PKC, protein kinase C; ROS, reactive oxygen species; SOD, superoxide dismutase; UGT1a6, glucuronosyltransferase-1a6.

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PM doses intermediate between protective and toxic amounts (15, 16). This led to the development of a hierarchical oxidative stress model, in which incremental PM doses induce protective and injurious effects (15, 16, 24). This model posits that at lower oxidative stress levels (tier 1), PM leads to the activation of a genetic antioxidant response element (ARE), resulting in the expression of phase II and antioxidant enzymes (11, 24). Activation of the ARE is dependent on the transcription factor, Nrf2 (11). Nrf2 induces the expression of heme oxygenase 1 (HO-1), NAD(P)H-quinone oxidoreductase (NQO1), GST, superoxide dismutase 3 (SOD3), and glucuronosyltransferase-1a6 (UGT-1a6) (25). These enzymes exert cytoprotective, antioxidant, and anti-inflammatory effects in lungs (11, 24–26). If this protection fails, escalation of oxidative stress leads to MAPK activation, cytokine/chemokine production, and inflammation (tier 2) (15, 16, 24). At the highest level of oxidative stress (tier 3), perturbation of the mitochondrial permeability transition pore and disruption of one-electron transfers lead to cellular apoptosis and necrosis (12, 27).

In addition to its role in heme catabolism (28), HO-1 has emerged as an important phase II and anti-inflammatory enzyme that is highly up-regulated by oxidative stress (29). This includes its expression in bronchial epithelial cells and macrophages during coincubation with DEP as well as organic extracts prepared from these particles (15). The active chemical compounds in these extracts include aromatic and polar substances, which can be extracted from DEP by silica gel chromatography (26). Both the aromatic fraction, which is enriched in PAHs, and the polar fraction, which is enriched in quinones, are capable of inducing oxidative stress (26). Based on the analogy with other pro-oxidative chemicals that induce HO-1 expression, it is possible that these functionalized DEP chemical compounds elicit HO-1 expression via activation of the b-ZIP transcription factor, Nrf2 (30). Oxidant-dependent regulation of Nrf2 activity is not completely understood and may occur at multiple levels (30–38). A well-accepted model proposes that under normal conditions, Nrf2 is anchored within the cytoplasm through its interaction with Keap1, a cytoskeleton-associated protein (31–34). By interfering with this interaction, oxidants are thought to deactivate cytoplasmic retention, resulting in nuclear localization of Nrf2, dimerization with other transcription factors, and, eventually, target gene activation (31–34). Additionally, several recent studies have shown that various oxidants induce Nrf2 expression by inhibiting its degradation by the ubiquitin-proteasome pathway (34–38). Finally, some agents may regulate Nrf2 expression at the level of gene transcription (38).

In this communication we explore the mechanism by which pro-oxidative DEP chemicals regulate Nrf2 and phase II enzyme expression in macrophages (RAW 264.7) and epithelial cells. We show that aromatic and polar DEP fractions induce mRNA expression of a number of phase II enzymes. We demonstrate that these responses are mediated through an effect on Nrf2 proteasomal regulation and nuclear accumulation. Finally, we show that concentrated ambient particulates act in the same way as the organic chemicals to induce HO-1 expression via Nrf2. These findings are important in understanding the lung defense against the pro-oxidative and proinflammatory effects of particulate pollutants and may help to characterize susceptible people who are more prone to develop asthma due to a defect in phase II enzyme expression.

Materials and Methods

Reagents

DMEM, penicillin-streptomycin, and L-glutamine were obtained from Invitrogen Life Technologies (Gaithersburg, MD). Bronchial epithelial growth medium was purchased from Cambrex (Walkersville, MD). F12-K medium was obtained from American Type Culture Collection (Manassas, VA). Type I rat tail collagen was purchased from Collaborative Research (Bedford, MA). FBS was purchased from Irvine Scientific (Santa Ana, CA). Anti-Nrf2 and anti-Keap1 Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HO-1 mAb was purchased from Stressgen (Victoria, Canada). Biotinylated swine anti-rabbit and rabbit anti-goat Abs were obtained from DakoCytomation (Carpinteria, CA). *N*-Acetylcysteine (NAC) and cyclohexamide (CHX) were obtained from Sigma-Aldrich (St. Louis, MO). MG-132 was purchased from Calbiochem (San Diego, CA). ECL reagents were purchased from Pierce (Rockford, IL). Effectene transfection reagent was obtained from Qiagen (Valencia, CA). The luciferase assay kit was purchased from Promega (Madison, WI). Ultraspec RNA was obtained from Biotecx (Houston, TX). AlexaFluor 594-conjugated goat anti-rabbit Ab and 4′,6-diamido-2-phenylindole hydrochloride (DAPI) were purchased from Molecular Probes (Eugene, OR). The iScript cDNA synthesis kit and iQ SYBR Green Supermix were obtained from Bio-Rad (Hercules, CA). Primers for traditional PCR were obtained from Invitrogen Life Technologies. All primers used for real-time PCR were purchased from E-Oligos (Hawthorne, NY). All organic solvents used were of Optima grade (Fisher Scientific, Pittsburgh, PA), and the solid chemicals were of analytical reagent grade.

Cell culture

The human bronchial epithelial cell line BEAS-2B, the murine bronchial epithelial cell line LA-4, and the murine macrophage cell line RAW 264.7 were obtained from American Type Culture Collection. BEAS-2B cells were cultured in bronchial epithelial growth medium in type I rat tail collagen-coated flasks or plates as previously described (15). LA-4 cells were grown in F12-K medium plus 15% FBS and 1% penicillin/streptomycin. RAW 264.7 were cultured in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% glutamine. All cell cultures were conducted in a 37°C humidified incubator supplied with 5% CO_2.

Preparation of DEP methanol extracts

Diesel exhaust particles were a gift from Dr. M. Sagai (National Institute of Environment Studies, Tsukuba, Ibaraki, Japan). These particles were collected from the exhaust in a 4JB1-type LD, 2.74 l, four-cylinder Isuzu diesel engine (Isuzu, Hokkaido, Japan) under a load of 10 torque onto a cyclone impactor equipped with a dilution tunnel constant volume sampler (12). DEP was collected on high capacity, glass-fiber filters, from which the scraped particles were stored as a powder in a glass container under nitrogen gas. The particles consist of aggregates in which individual particles are <1 μm in diameter. The chemical composition of these particles, including PAH and quinone analysis, was previously described (26). DEP methanol extracts were prepared as previously described (12). Briefly, 100 mg of DEP were suspended in 25 ml of methanol and sonicated for 2 min. 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. The tube was reweighed to determine the amount of methanol-extractable DEP components. Dried DEP extract was then dissolved in DMSO at a concentration of 100 μg/μl. The aliquots were stored at −80°C in the dark until use.

Preparation of DEP fractions

DEP (1.2 g) was extracted by sonication with 200 ml of methylene chloride. The extract was filtered using a Millipore filtration system (Bedford, MA) with a 0.45-μm pore size nylon filter. Preparation of DEP fractions was conducted as previously described with some modifications (26). The methylene chloride extract was concentrated by rotary evaporation and asphaltenes (insoluble, polar chemicals with S and O heteroatoms) were precipitated by adding 25 ml of hexane and shaking. The contents were left overnight in the freezer and centrifuged, and the supernatant hexane was collected. The precipitate was washed twice with hexane, and the washings were combined with the first hexane extract, concentrated, and dried over anhydrous sodium sulfate. The extract thus prepared was subjected to gravity-fed, silica gel column chromatography. Three 1.5 × 50-cm columns were packed with 26 g of activated silica gel between 1 cm of anhydrous sodium sulfate and conditioned with hexane. The extract was split into three equal aliquots and then applied to each column. Aliphatic, aromatic, and polar fractions were collected by successive elution with 70 ml of hexane, 150 ml of hexane/methylene chloride (3/2, v/v), and 90 ml of methylene chloride/methanol (1/1, v/v), respectively. The flow rate was 1.5 ml/min. The elution of the aromatic fraction was monitored by an UV lamp at long wavelength (365 nm). The eluates from the three columns were combined and concentrated by roto-evaporation and made up to 1 ml in a 4-ml graduated vial, the aliphatic fraction in hexane and the others in methylene chloride. The vials were tightly sealed with a silicone-lined cap and stored in −80°C until further use. The weight of the fractions was determined in a microbalance after evaporating off the hexane or methylene chloride from a known sample volume. Alkanes in the aliphatic fraction were characterized

by a GC (Varian 3400; Palo Alto, CA) with a Structure Probe (West Chester, PA) injector) equipped with a flame ionization detector and a DB-5 column (30 m, 0.25 mm inside diameter, 0.25-μm film). The fractions were dried with N_2 gas and redissolved in DMSO for in vitro biological studies.

Elemental carbon (EC) and OC measurements

The EC and OC contents of PM were measured from a 1-cm^2 punch taken from quartz-fiber filters using a thermo-optical transmittance analyzer (TOT; Sunset Laboratory, Tigard, OR), according to the procedure set by the National Institute for Occupational Safety and Health (39).

PAH and quinone measurements

PAH content in the initial extract and in each fraction was determined by HPLC fluorescence with selective excitation/emission conditions. The method was optimized for the identification and quantification of the 16 PAHs classified by the Environmental Protection Agency as hazardous pollutants (40). Quinone content was analyzed using the method described by Cho et al. (41). Briefly, quinones in the samples were analyzed in their most stable diacetyl derivatives quantitated by gas chromatography/mass spectrometry. Deuterated internal standards were added before extraction and derivatization. One hundred milligrams of zinc, anhydrous tetrahydrofuran, and 200 μl of acetic anhydride were added to samples. After heating at 80°C for 15 min, samples were cooled to room temperature, and an additional 100 mg of zinc was added, followed by an additional 15-min heating. The reaction was quenched with 0.5 ml of water and 3 ml of pentane. After centrifugation at 750 $\times$ *g* for 10 min, the pentane layer was evaporated to dryness, and the residue was reconstituted in 50–100 μl of dry acetonitrile. 1,2-Naphthoquinone, 1,4-naphthoquinone, phenanthrenequinone, and anthraquinone were analyzed by the electron impact gas chromatography/mass spectrometry technique using a mass selective detector equipped with an automatic sampler (Hewlett-Packard, Palo Alto, CA) (41).

Ambient particle collection

Ambient coarse (2.5–10 μm) and ultrafine ($<$0.15 μm) particles were collected in the Los Angeles basin during the period of May 2003, using a particle concentrator as described by Li et al. (7). These samples were collected at Downey, an area in southeastern Los Angeles, impacted mostly by vehicular emissions. To determine particle mass and chemical composition, parallel samples were collected on Teflon and quartz filters with a Micro Orifice Uniform Deposit Impactor (MOUDI; MSP, Shoreview, MN) as previously described (7). We used Teflon filters to determine the mass and metal and trace element contents by x-ray fluorescence and quartz filters to determine the inorganic content (sulfate and nitrate) as well as organic carbon (using a MnO_2-catalyzed CO_2 formation).

DTT assay

The DTT assay was used to quantitate the redox activity of PM as previously reported (7, 42). This assay measures DTT oxidation by quinones in the following net reaction: DTT + $2O_2 \rightarrow$DTT-disulfide + $2O_2^-$. Briefly, the crude DEP extract or its fractions were incubated with 100 μM DTT in a phosphate buffer at pH 7.4 for 15–60 min. Aliquots of the incubation mixture were mixed with Tris buffer, pH 8.9, and the 5,5′-dithiobis-(2-nitrobenzoic acid) solution, and the OD was read at 412 nm.

Preparation of cell lysates

Whole lysates of RAW 264.7 cells were prepared as previously described (26). To prepare nuclear extracts, RAW 264.7 cells were collected by scraping in cold PBS. The cell pellet was lysed in 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF, and 0.3% Nonidet P-40. Nuclear proteins were then extracted using a buffer containing 25% glycerol, 20 mM HEPES, 0.6 M KCl, 1.5 mM $MgCl_2$, and 0.2 mM EDTA. Protein concentrations were determined using the Bradford method.

Western blotting analysis

Western blotting was conducted as previously described (26). One hundred to 150 μg of total protein was separated by SDS-PAGE before transfer to polyvinylidene difluoride membranes. HO-1 protein was detected by anti-HO-1 mAb at 0.3 μg/ml and rabbit anti-mouse Ab conjugated to HRP according to the manufacturer's instructions. Anti-Nrf2 Ab was used at 0.6 μg/ml. Biotinylated swine anti-rabbit Ab (1/1,000) was used as secondary Ab, followed by HRP-conjugated avidin-biotin complex (1:10,000). Anti-Keap1 Ab was used at 0.6 μg/ml, followed by biotinylated rabbit anti-goat Ab as secondary Ab. HRP-conjugated avidin-biotin complex was used at 1/10,000 dilution. Blots were developed with the ECL reagent according to the manufacturer's instruction.

RT, classical, and real-time PCR analyses

Total RNA was extracted using Ultraspec RNA according to manufacturer's instructions. RT was performed at 42°C in a total volume of 20 μl containing 5 μg of total RNA; 0.5 μg of oligo$(dT)_{12–18}$; 10 mM DTT; 0.5 mM each of dATP, dGTP, dCTP, and dTTP; and 10 U of Moloney leukemia virus reverse transcriptase (26). HO-1 primers for PCR amplification of a 668-bp mouse HO-1 fragment (26) were obtained from Invitrogen Life Technologies. The primer sequences of mouse HO-1 are 5′-CTGTGTAACC TCTGCTGTTCC-3′ and 5′-CCACACTACCTGAGTCTACC-3′ (26). The sequences of mouse β-actin primers are 5′-TGGAATCCTGTGGCA TCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ (26). The sequences for mouse Nrf2 primers are 5′-TCTCCTCGCTG GAAAAAGAA-3′ and 3′-AATGTGCTGGCTGTGCTTTA-5′ (43). PCRs for HO-1, Nrf2, and β-actin were performed in a total reaction volume of 25 μl containing 4 μl of cDNA template, 0.5 μM sense and antisense primers, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 2.5 U of *Taq* DNA polymerase in a PerkinElmer thermal cycler (Norwalk, CT). Samples were heated to 95°C for 2 min and subjected to 40 cycles of amplification (1 min at 94°C, 1 min at 58°C, and 1 min at 72°C), followed by 10 min at 72°C for final extension. PCR products were electrophoresed in 2% agarose gels and viewed by ethidium bromide staining.

For real-time PCR analysis, RAW 264.7, LA-4, and BEAS-2B cells were treated with 25 μg/ml crude DEP extract for 6 and 3 h, respectively, before RNA extraction. Total RNA was isolated as described above, and cDNA was generated using the iScript cDNA synthesis kit. Real-time PCR was performed with iQ SYBR Green Supermix using PCR primers in an iCycler (Bio-Rad). The primers for real-time PCR are listed in Table I. Relative mRNA expression was determined by the Pfaffl equation.

Table I. *Primer sequences for real-time PCR analyses*

Genes	Species	Forward Primer	Reverse Primer
Actin	Mouse	AGCCATGTACGTAGCCATC	CTCTCAGCTGTGGTGGTGA
Nrf2		CTCGCTGGAAAAAGAAGTG	CCGTCCAGGAGTTCAGAGG
HO-1		CACGCATATACCCGCTACCT	CCAGAGTGTTCATTCGAGA
Catalase		GAGACCTGGGCAATGTGAT	GTTTACTGCGCAATCCCAAT
SOD2		GCGGTCGTGTAAACCTCAT	CCAGAGCCTCGTGGTACTTC
SOD3		CTGAGGACTTCCCAGTGAC	GGTGAGGGTGTCAGAGTGT
NQO1		TTCTCTGGCCGATTCAGAGT	GGCTGCTTGGAGCAAAATG
GST Ya		CGCCACCAAATATGACCTCT	CCTGTTGCCCACAAGGTAGT
UGT-1a6		GCAACCAGTTTGTTCCTGGT	AGACAGAGGGCAGGTTGAA
GPx		GTCCACCGTGTATGCCTTCT	TCTGCAGATCGTTCATCTCG
GAPDH		AACTTTGGCATTGTGGAAG	ACACATTGGGGGTAGGAAA
GR		CAACACAGTGGCCATTCAC	TTGTTTCTCATGGACCACCA
GCLS		TGGAGCAGCTGTATCAGTG	AGAGCAGTTCTTTCGGGTA
Actin	Human	GGACTTCGAGCAAGAGATG	AGCACTGTGTTGGCGTACG
HO-1		TCCGATGGGTCCTTACACTC	TAAGGAAGCCAGCCAAGAA
Nrf2		GCGACGGAAAGAGTATGAC	GTTGGCAGATCCACTGGTTT

RAW 264.7 transfection with SX2 plus wild-type or dominant negative Nrf2 and luciferase assay

RAW 264.7 cells were plated at 2×10^5 cells/well in a 12-well plate 24 h before transfection. Cells were transfected with the Effectene transfection reagent according to the manufacturer's recommendations. Each well was transfected with a DNA mixture containing 100 ng of pSX2-luc, 50 ng of pCMV-β-galactosidase, and 50 ng of empty vector or the Nrf2 expression plasmid (either wild-type or dominant negative mutant). The luciferase activity in each sample was corrected for variations in transfection efficiency with the corresponding β-galactosidase activity. Luciferase activity was measured using a luciferase reagent kit from Promega (Madison, WI).

Confocal microscopy

Confocal microscopy was performed as previously described (44). RAW 264.7 cells were treated with 50 μg/ml DEP extract for 2 h before washing in DMEM and attachment of 10^6 cells to each polylysine-coated coverslip. Cells were fixed in 4% paraformaldehyde in 30 mM sucrose for 10 min at room temperature, followed by 10 min in 50 mM NH_4Cl to neutralize aldehyde groups. For intracellular staining, coverslips were permeabilized with 0.1% Triton X-100/PBS for 30 min. After blocking in 1% BSA plus 1% normal goat serum, cells were incubated with polyclonal anti-Nrf2 Ab (1/200) for 16 h. After washing in 0.1% Triton X-100/PBS, slides were incubated with Alexa 594-conjugated goat anti-rabbit Ab for 60 min, followed by cellular nuclear staining with DAPI. Control slides were stained with rabbit nonimmune serum before addition of the secondary Ab. Samples were examined under a Leica inverted TCS-SP confocal microscope (Deerfield, IL), using $\times$100 and $\times$43 objectives, respectively. Data acquisition was accomplished with Leica confocal software.

Preparation of thioglycolate-elicited peritoneal macrophages from Nrf2$^{-/-}$ mice

Nrf2$^{-/-}$ animals on a mixed C57BL6/SV129 background were initially obtained from Dr. Y. Kan (University of California, San Francisco, CA) (45) and enriched 87.5% on a C57BL6 background by three rounds of breeding. *Nrf2*$^{-/+}$ littermate controls and wild-type C57BL/6 mice were used as comparative controls. Peritoneal macrophages were collected by i.p. instillation of thioglycolate for 5 days before harvesting. Peritoneal macrophages were lavaged from the peritoneal cavity in complete DMEM, and 10^6 cells/well were used for stimulation with 50 μg/ml crude DEP extract. The macrophages were then lysed and processed for HO-1 blotting as described above.

Results

Organic DEP extracts induce mRNA expression of phase II enzymes in BEAS-2B and RAW 264.7 cells

We have previously demonstrated that organic DEP extracts induce HO-1 expression in PM targets (macrophages and epithelial cells) (16). This cytoprotective response is dependent on the activation of an ARE in the HO-1 promoter (26). In addition to HO-1, other phase II enzymes (e.g., HO-1, NQO1, GST, catalase, SOD, and glutamylcysteine synthase) are induced by the ARE in the lung (25). Besides their role in antioxidant defense, these enzymes detoxify electrophilic chemicals such as organic peroxides, lipid peroxides, epoxides, and quinones (11). To determine which of the above phase II enzymes are induced by organic DEP chemicals, a real-time PCR approach was used to study message expression in RAW 264.7 and LA-4 cells (Fig. 1). We observed a statistically significant increase in HO-1, SOD3, NQO1, GST-Ya, and UGT-1a6 mRNA expression in RAW 264.7 cells exposed the crude DEP extract (Fig. 1*A*). All the above phase II enzymes have been shown to be regulated by the transcription factor, Nrf2 in vitro and in vivo (Table II). In contrast, there was no increase in mRNA expression for catalase, SOD2, and glutathione peroxidase (Fig. 1*A*). There was also no increase in the expression of the housekeeping genes, actin and GAPDH (Fig. 1*A*). In a similar fashion, the messages of HO-1, GST-Ya, UGT1a6, and γ-glutamate cysteine ligase regulatory subunit were significantly increased in LA-4 cells exposed to the crude DEP extract, whereas catalase and glutathione reductase mRNA levels remained unchanged (Fig. 1*B*).

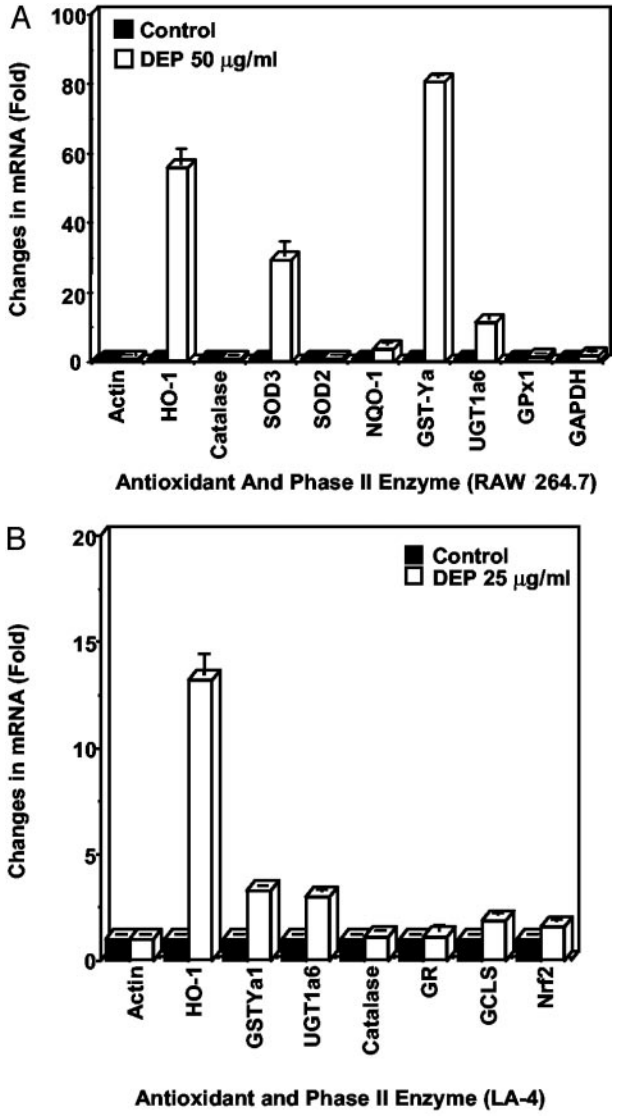

FIGURE 1. Real-time PCR analysis of phase II enzyme expression by a crude DEP extract. *A*, Real-time PCR analysis in RAW 264.7 cells. Cells were stimulated with 50 μg/ml DEP extract for 6 h before processing for mRNA analysis. RT and real-time PCR analyses were performed as described in *Materials and Methods*. The data were reproduced in a separate experiment. *B*, Real-time PCR analysis in LA-4 cells. Cells were exposed to 25 μg/ml DEP extract for 3 h before processing for PCR analysis.

Establishment of a stable transfected cell line to test the effect of pro-oxidative DEP chemicals on ARE activation

DEP contain a host of organic chemicals, among which the aliphatic hydrocarbons, heterocyclic compounds, aromatic hydrocarbons (e.g., PAH), and polar compounds (e.g., quinones) constitute

Table II. *Evidence demonstrating the regulatory role of Nrf2 in ARE-dependent expression of antioxidant and phase II enzyme genes*

Gene	Ref. no.
HO-1	25, 30, 45
NQO-1	25, 45–47
GCLS	25, 45
GST	25, 31, 47–50
UGT1a6	25, 45, 51
SOD3	25

the major groups (52–55). Previous studies have demonstrated that the polar and, to a lesser extent, the aromatic fractions are inducers of HO-1 expression in RAW 264.7 cells (26). To provide a rapid and reproducible readout of ARE activity, we constructed a RAW 264.7 line with stable expression of an ARE-luciferase reporter gene. This cell line exhibited a dose-dependent increase in luciferase activity in response to the crude DEP extract (Fig. 2*A*) and was therefore suitable for comparing the aliphatic, aromatic, and polar chemical fractions. The amounts and recovery of those fractions during fractionation of a crude DEP extract by silica gel

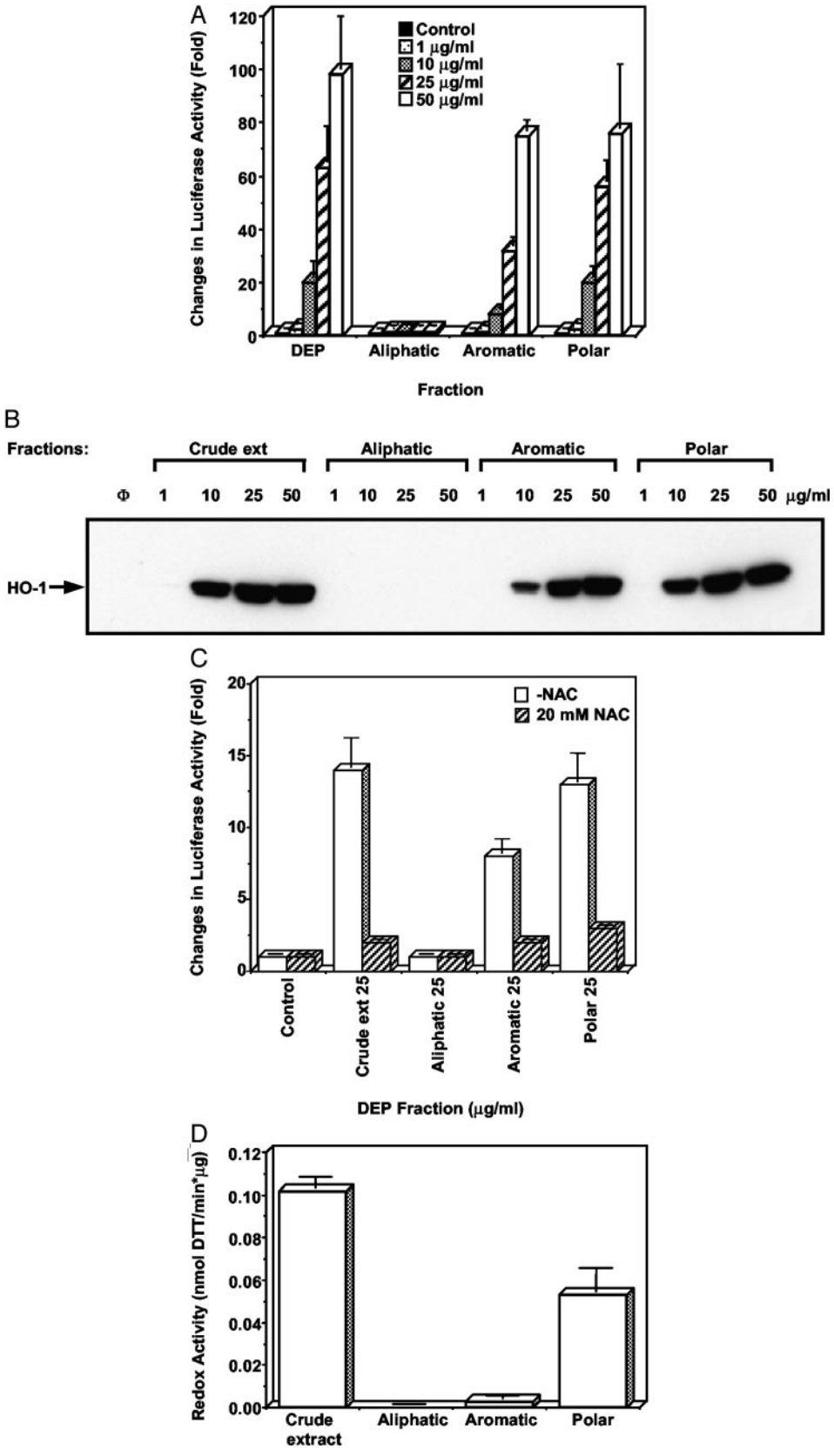

FIGURE 2. Activation of the ARE and HO-1 expression in RAW 264.7 cells by redox-active DEP chemicals. *A*, Reporter gene assay showing differential effects of the aliphatic, aromatic, and polar chemicals on ARE activation. RAW 264.7 cells were stably transfected with a plasmid containing two copies of the consensus ARE sequence in the HO-1 promoter linked to a luciferase reporter. Cells were stimulated with the crude DEP extract or its fractions for 6 h at the indicated concentrations. Values represent the mean ± SD of three separate experiments. *B*, Western blotting to assess HO-1 expression by DEP fractions. RAW 264.7 cells were treated for 6 h with the indicated concentrations of the chemicals before harvesting. One hundred micrograms of cellular lysate was resolved by 10% SDS-PAGE, transferred to the polyvinylidene difluoride membrane, and immunoblotted with monoclonal anti-HO-1 Ab. *C*, NAC inhibits ARE activation. Stable transfected RAW 264.7 were treated with the DEP extract or its fractions for 6 h in the absence or the presence of 20 mM NAC before luciferase assays. The values represent the mean ± SD from two separate culture wells, each measured in duplicate. *D*, Analysis of in vitro DEP redox capacity. The redox cycling activity of DEP fractions was analyzed using the DTT assay as described in *Materials and Methods*.

Table III. *Recovery of major organic fractions from 1 g of DEP*

Fraction	Elution Solvent	Amount (mg)	Recovery (%)[a]
Aliphatic	Hexane	235	23.5
Aromatic	Hexane/methylene chloride (3/2, v/v)	105	10.5
Polar	Methylene chloride/methanol (1/1, v/v)	100	10.0
Total		440	44.0

[a] The amount of asphaltene from 1 g of DEP = 289.7 mg, which represents 29% of particle mass.

Table V. *Quinone content in DEP fractions*[a]

Quinones	Quinone Content in DEP Fractions (μg/g DEP)			
	Crude extract	Aliphatic	Aromatic	Polar
1,2 NQ	22.34	ND	ND	2.28
1,4 NQ	19.94	ND	ND	6.91
9,10 PQ	18.73	ND	ND	6.03
9, 10 AQ	69.34	ND	ND	36.86

[a] Quinone contents in crude DEP extract and fractions were analyzed as described in *Materials and Methods*. Four standard quinones were used for quantitation: 1,2 NQ, 1,2-naphthoquinone; 1,4 NQ, 1,4-naphthoquinone; 9,10 PQ, 9,10-phenanthrenequinone; and 9,10 AQ, 9,10-athraquinone. ND, not detected.

chromatography are shown in Table III (26). Chemical analysis confirmed that the aromatic fraction contained an abundance of PAHs (Table IV), whereas the polar fraction lacked PAHs but was enriched with quinones (Table V; 26). Although exposure to the aliphatic fraction lacked any effect, the aromatic and polar chemical compounds induced a dose-dependent increase in luciferase activity (Fig. 2*A*). The polar was more active than the aromatic fraction on a dose-by-dose comparison (Fig. 2*A*). To confirm the biological relevance of the reporter gene assay, we showed that endogenous HO-1 expression by the polar fraction is more robust than the aromatic fraction (Fig. 2*B*). The aliphatic fraction was inactive (Fig. 2*B*).

ARE-mediated gene expression by heavy metals and pro-oxidative chemicals is dependent on electrophilic chemistry and protein thiol modification (11). This includes modification of thiol groups that control the function of the ARE-inducing transcription factor, Nrf2 (14, 56). Thiol modification is important in understanding the biological effect of DEP chemicals, as demonstrated by the ability of NAC to interfere in HO-1 expression (12, 15). This agent uses its Src homology group to directly neutralize electrophilic DEP chemicals (11, 24). It is of interest, therefore, that the NAC inhibitory effect also applies to the actions of the aromatic and polar chemical fractions (Fig. 2*C*). NAC also suppressed ARE-luciferase activity by the crude DEP extract (Fig. 2*C*).

As well as participating in electrophilic interactions, DEP engage in redox cycling reactions that lead to ROS production; this effect may also be relevant to the activation of the ARE (12, 26). We have modified an in vitro assay developed by Kumagai et al. (42) that directly measures the capacity of intact particles and their organic compounds to redox cycle in the presence of DTT (see *Materials and Methods*). Quinones and possibly other redox cycling chemicals lead to DTT oxidation and O_2^- generation (7, 42). When tested in the DTT assay, the polar fraction was considerably more potent than the aromatic fraction, whereas aliphatic material was inactive (Fig. 2*D*). These results suggest that the increased potency of the polar fraction in the activation of the ARE may be explained by its increased content of redox cycling chemicals.

Table IV. *PAH content in each DEP fraction*[a]

PAH	PAH Content (μg/g DEP)			
	Crude extract	Aliphatic	Aromatic	Polar
NAP	89	0.04	3.71	0.00
ACE	67	0.00	0.48	0.00
FLU	153	0.00	6.18	0.00
PHE	1576	0.04	110.87	0.00
ANT	24	0.00	0.95	0.12
FLT	678	0.00	45.13	1.01
PYR	530	0.00	23.37	0.05
BAA	91	0.00	6.18	0.00
CRY	158	0.00	7.7	0.00
BBF	48	0.00	1.71	0.00
BKF	20	0.00	0.29	0.00
BAP	16	0.00	0.00	0.00
DBA	16	0.00	0.10	0.00
BGP	18	0.00	0.10	0.00
IND	18	0.00	0.10	0.00

[a] PAH content was measured as previously described (40). Sixteen standard PAH were used to quantitate the PAH content in each fraction. NAP, naphthalene; ACE, acenaphthalene; FLU, fluorene; PHE, phenanthrene; ANT, anthracene; FLT, fluoranthene; PYR, pyrene; BAA, benzo(a)anthracene; CRY, chrysene; BBF, benzo(b)fluoranthene; BKF, benzo(k)fluoranthene; BAP, benzo(a)pyrene; DBA, dibenz(a,h)anthracene; BGP, benzo(ghi)perylene; IND, indeno(1,2,3,-cd)pyrene.

Activation of the ARE by the organic DEP extract is dependent on Nrf2

Nrf2 together with small Maf and AP-1 transcription factors are involved in the activation of ARE and phase II enzyme expression by electrophilic chemicals (30–33). To directly demonstrate the role of Nrf2 in the activation of the ARE by DEP chemicals, RAW 264.7 cells were transiently transfected with a cDNA construct containing the SX2 domain of the HO-1 gene fused to a luciferase reporter (SX2-Luc) (Fig. 3*A*). This enhancer region contains three AREs (26). Treatment with a crude DEP extract induced a 5.3-fold increase in luciferase activity (Fig. 3*A*). Cotransfection of the reporter gene with a wild-type Nrf2 construct, induced a 7.5-fold increase in luciferase activity. This response was minimally enhanced by DEP chemicals (Fig. 3*A*). In contrast, cotransfection with a dominant negative Nrf2 construct almost totally suppressed the DEP response, showing that Nrf2 is required for transcriptional activation (Fig. 3*A*). Transfection efficiency was normalized using β-galactosidase activity. The same result was obtained when RAW 264.7 cells were transfected with small interfering RNAs, which suppress endogenous Nrf2 expression (data not shown).

The in vivo relevance of Nrf2 transcriptional activity in HO-1 expression by pro-oxidative DEP chemicals was confirmed using peritoneal macrophages from *Nrf2*$^{-/-}$ mice (45). Compared with basal HO-1 expression in wild-type and *Nrf2*$^{-/+}$ macrophages, this phase II enzyme was not expressed in *Nrf2*$^{-/-}$ macrophages under resting conditions (Fig. 3*B*). Moreover, after the addition of a crude DEP extract, there was considerably less HO-1 expression in *Nrf2*$^{-/-}$ compared with *Nrf2*$^{-/+}$ and wild-type animals (Fig. 3*B*).

To determine whether DEP chemicals stimulate the ARE by promoting Nrf2 translocation to the nucleus, we used anti-Nrf2 Abs and confocal microscopy to visualize the subcellular distribution of Nrf2. In resting cells, Nrf2 was present at low levels and was detected as a finely speckled pattern (Fig. 4). DEP treatment caused a dramatic increase in Nrf2 staining intensity. Although most of the protein was randomly distributed in resting cells, the distribution was changed to a clumped staining pattern in the nucleus of DEP-treated cells (Fig. 4). This resulted in a composite

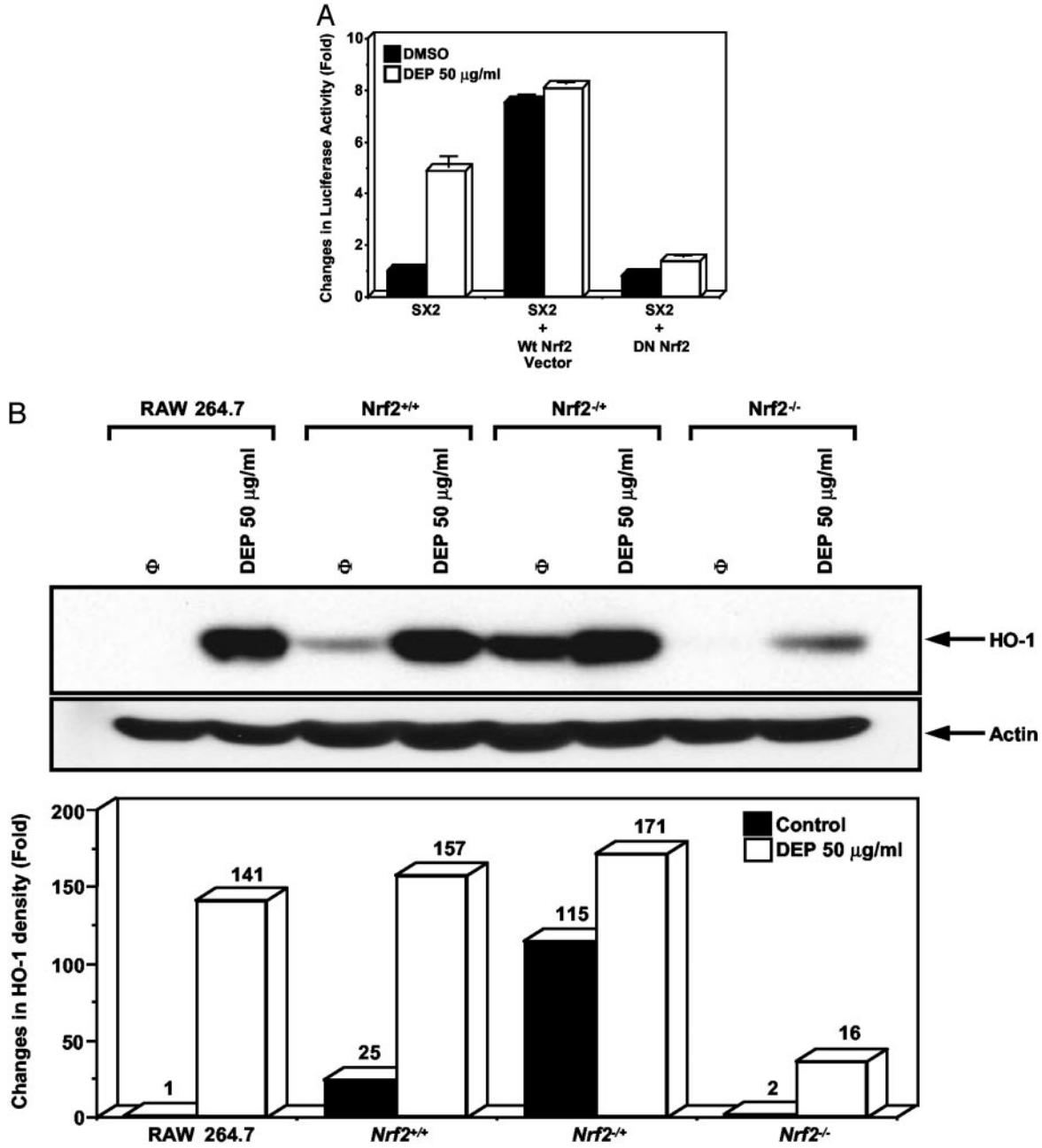

FIGURE 3. Nrf2 is required for the activation of the ARE and HO-1 induction. *A*, The inhibitory effect of dominant negative Nrf2 on ARE activation. RAW 264.7 cells were transiently transfected with the HO-1 enhancer (SX2) linked to luciferase, as well as either a wild-type (Wt) or a dominant negative (DN) Nrf2 vector. ARE activity was determined by a luciferase reporter assay as described in Fig. 2. β-Galactosidase activity was used to normalize luciferase activity. *B*, Induction of HO-1 expression in peritoneal macrophages from wild-type, $Nrf2^{-/+}$, and $Nrf2^{-/-}$ mice. Peritoneal macrophages were obtained as described in *Materials and Methods*. Cells were plated at 10^6/well in complete DMEM in six-well plates and stimulated with either DMSO or DEP extract (50 μg/ml) for 6 h. Twenty micrograms of total cellular protein was used for SDS-PAGE. HO-1 expression was determined as described in Fig. 2.

pink staining pattern observed when the Nrf2 and DAPI (nuclear) fluorescence panels were superimposed (Fig. 4).

The enhanced Nrf2 staining in stimulated cells suggests that DEP induces Nrf2 expression. This induction was confirmed by immunoblotting analysis of cellular and nuclear lysates (Fig. 5). In a whole cell extract we demonstrated a dose-dependent increase in Nrf2 protein expression by the crude DEP extract as well as in response to the aromatic and polar chemical groups (Fig. 5*A*). The aliphatic fraction was inactive (Fig. 5*A*). This increase was specific for Nrf2 because there was no change in actin expression (Fig. 5*A*, *bottom panel*). In accordance with its effects on the ARE (Fig. 2), the polar was more potent than the aromatic fraction in eliciting Nrf2 expression (Fig. 5*A*, *middle panel*). A similar trend was observed when nuclear extracts were used, namely, increased Nrf2 expression by the aromatic and polar fractions, with the latter being more active (Fig. 5*B*, *upper left panel*). In contrast, there was no change in Nrf2 accumulation in the nucleus with the aliphatic fraction. The level of histone H1 did not change appreciably in response to any treatment (Fig. 5*B*, *bottom panels*). Interestingly, NAC treatment interfered in the nuclear accumulation of Nrf2 (Fig. 5*B*, *upper right panel*), which is in accordance with its effect on the activation of the ARE (Fig. 2*C*).

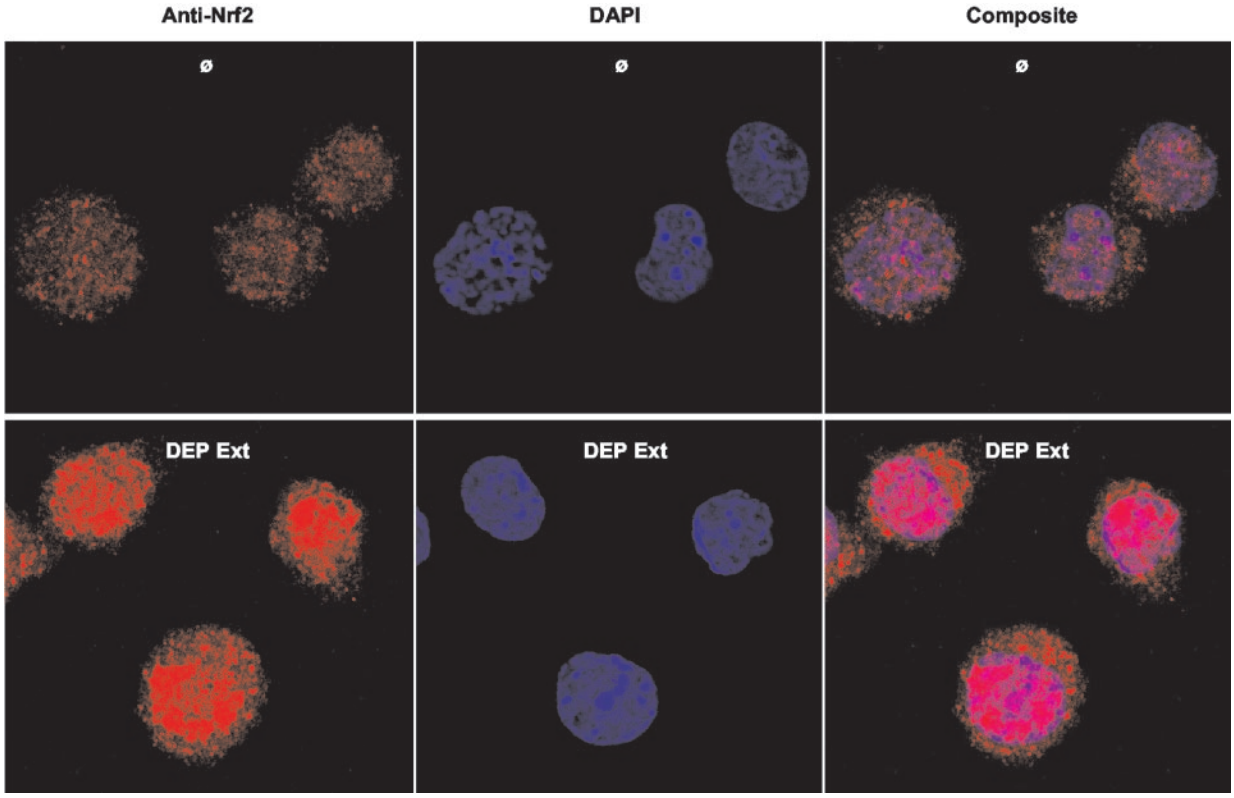

FIGURE 4. Confocal microscopy to show intercellular Nrf2 expression. RAW 264.7 cells were treated with the crude DEP extract for 2 h. Confocal microscopy was conducted as described in *Materials and Methods*. Nrf2 was stained with polyclonal anti-Nrf2 Ab, followed by Alexa 594-conjugated goat anti-rabbit secondary Ab, whereas the nucleus was counterstained with DAPI.

Nrf2 abundance is post-translationally regulated

To investigate the mechanism by which DEP chemicals induce Nrf2 expression, we monitored the temporal accumulation of Nrf2 protein and mRNA after cellular stimulation. An increase in the level of Nrf2 protein was detected within 20 min after treatment and was maintained for up to 6 h, the last time point tested (Fig. 6*A*). Interestingly, this increase in Nrf2 protein was not accompanied by increased mRNA expression at any time point (Fig. 6*B*). By contrast, HO-1 mRNA was increased by the same stimulus (Fig. 6*B*), and this preceded the increase in protein expression at 4 h (Fig. 6*A*). The initial appearance of the HO-1 message (<30 min) is compatible with the prior accumulation (<20 min) of Nrf2 protein (Fig. 6, *A* and *B*). DEP also stimulated Nrf2 protein expression without affecting mRNA levels in BEAS-2B cells (Fig. 6*C*). In contrast, expression of Keap1 protein (Fig. 6*A*) or β-actin mRNA/protein (Fig. 6) did not change in response to the chemical exposure (Fig. 6*A*). Taken together, these results suggest that DEP regulates Nrf2 expression by post-transcriptional mechanisms.

DEP chemicals increase Nrf2 protein half-life by affecting proteasomal degradation

It has recently been reported that pro-oxidative chemicals, including β-naphthoflavone and cadmium, increase Nrf2 expression by interfering in proteasomal degradation in HepG2 and Hepa cells (34, 38). To determine whether Nrf2 is similarly regulated in RAW 264.7, cells were treated with MG-132, a 26S proteasome inhibitor, and whole cell lysates were subjected to Nrf2 immunoblotting. MG-132 induced a dose-dependent increase in Nrf2 protein abundance without requiring a costimulus (Fig. 7*A*). Similar results were obtained with another 26S proteasome inhibitor, lactacystin (not shown). We next determined the half-life of Nrf2 after inhibition of protein synthesis by CHX. Cells treated with the DEP extract, followed by CHX exposure, showed a rapid disappearance of the accumulated Nrf2 protein, with an estimated half-life of ~30 min (Fig. 7*B*). The addition of MG-132 to CHX prolonged the Nrf2 half-life to ~90 min (Fig. 7*B*). Due to the low abundance of Nrf2 in resting cells, it was not possible to estimate protein half-life in the absence of DEP chemicals. However, we could show that combining the DEP extract with CHX prolonged Nrf2 expression in MG-132-treated cells (Fig. 7*C*). This suggests that the crude extract enhanced the inhibitory effects of MG-132 on Nrf2 proteasomal degradation. Analogous experiments with the aromatic and polar chemical fractions showed that similar to the crude extract, these materials could induce Nrf2 expression with a half-life of ~30 min (Fig. 7*D*). These results are consistent with previously published reports that assessed Nrf2 protein half-life through the use of CHX and either immunoblotting or [^{35}S]methionine pulse labeling (34).

Ambient particulate matter activates the ARE and induces HO-1 expression via Nrf2

We asked whether the engagement of the antioxidant defense pathway by organic DEP chemicals apply to "real-life" ambient PM. Ambient PM consists of different particle sizes: coarse PM (PM_{10}) are typically derived from soil, road dust, construction debris, or aggregation of smaller combustion particles, whereas ultrafine (<0.1 μm) PM are derived from the combustion of fossil fuel products, including diesel, as well as nucleation processes (54). A comparison of the effects of ambient ultrafine and coarse particles collected by particle concentrators in the Los Angeles basin demonstrated that the ultrafines were more potent than PM_{10} in generating oxidative stress (7). This outcome is clearly related to the

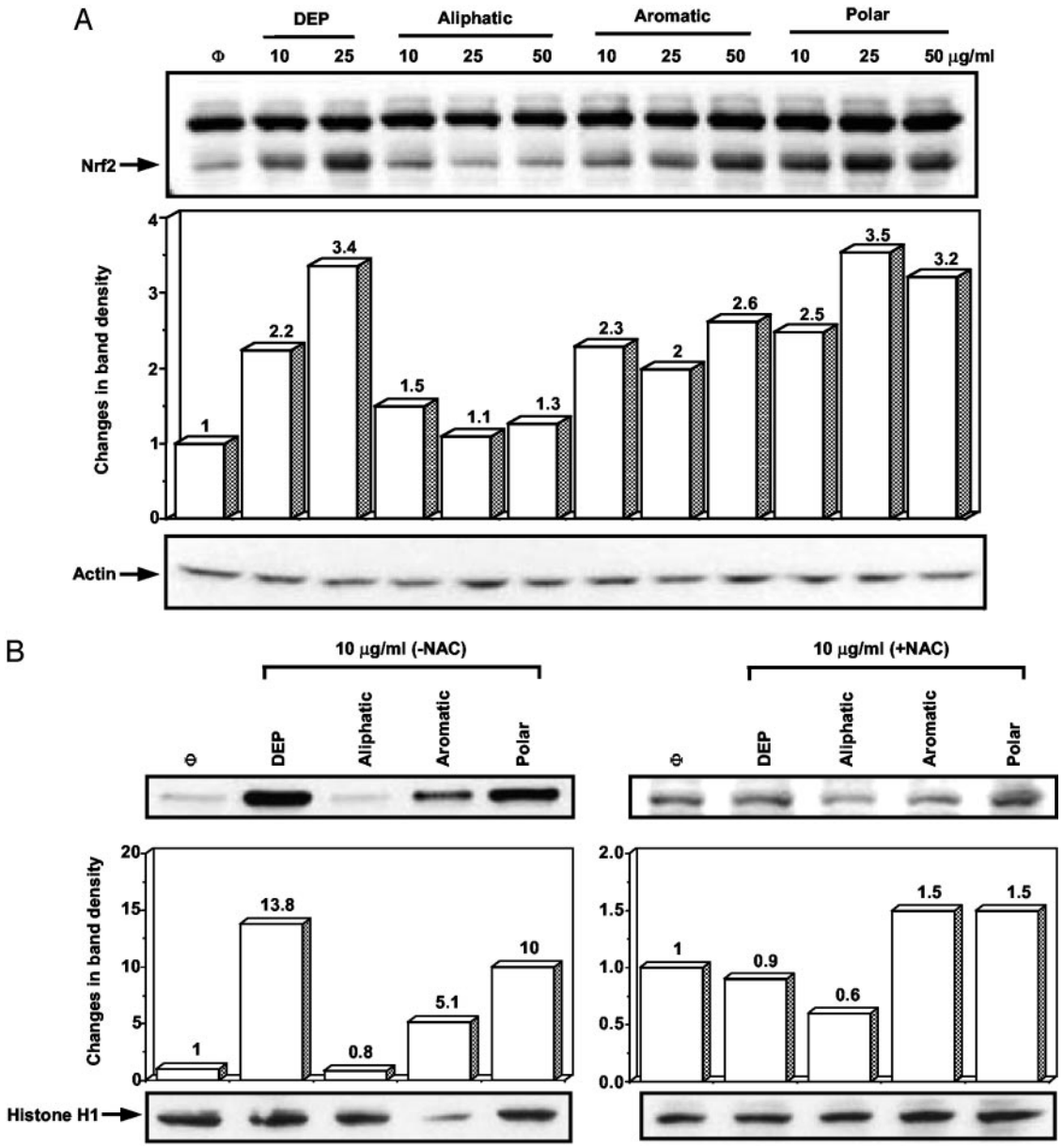

FIGURE 5. DEP induced Nrf2 protein nuclear accumulation. *A*, Whole cell Nrf2 expression in response to different chemical fractions. Cells were treated with the indicated amounts of the crude DEP extract or the individual chemical fractions for 1 h. Whole cell lysates were used for Nrf2 immunoblotting as described in *Materials and Methods*. Scanning density was performed using laser Personal Densitometer SI and ImageQuant software (both from Amersham Biosciences). *B*, Nuclear Nrf2 accumulation and the effect of NAC. Cells were preincubated with NAC (20 mM) or carrier for 2 h, followed by exposure to the various chemicals for 1 h. Nuclear proteins were extracted and used for immunoblotting with polyclonal anti-Nrf2 or anti-histone H1 Abs. The results were reproduced twice.

small size, large surface area, and high PAH content of ultrafine particles (7). Exposure of RAW 264.7 cells to PM_{10} and ultrafine particles led to induction of HO-1 expression at concentrations as low as 10 μg/ml, with ultrafines being more potent (Fig. 8*A*). Indeed, ultrafine particles used at 24 μg/ml were as potent as a comparable dose of DEP (Fig. 8*A*). Moreover, an examination of particle effects on Nrf2 protein accumulation (Fig. 8*B*) and activation of the ARE (Fig. 8*C*) confirmed that the ultrafines were more potent than the coarse particles. Analysis of OC and EC carbons, nitrates, sulfates, metals, and elements, showed that whereas trace elements and metals are the major components of the coarse particles, the ultrafine particles contained a 4.6-fold higher content of OC than the coarse particles (Table VI). We have previously shown that the increased OC content is accompanied by an increased PAH content (7). In addition, in vitro analysis of particle redox activity using DTT assay demonstrated that the ultrafine particles are more active than the coarse particles (Fig. 8C, *inset*). This is consistent with our previous findings that the induction of HO-1 by ambient particles correlates with their PAH content and in vitro redox activity (7).

Discussion

We demonstrate that DEP chemicals and ambient PM induce the expression of phase II enzymes by perturbing Nrf2 stability and ARE transcriptional activity. The induction of these antioxidant and detoxification pathways in macrophages and epithelial cells is dependent on aromatic and polar chemical groups, which through

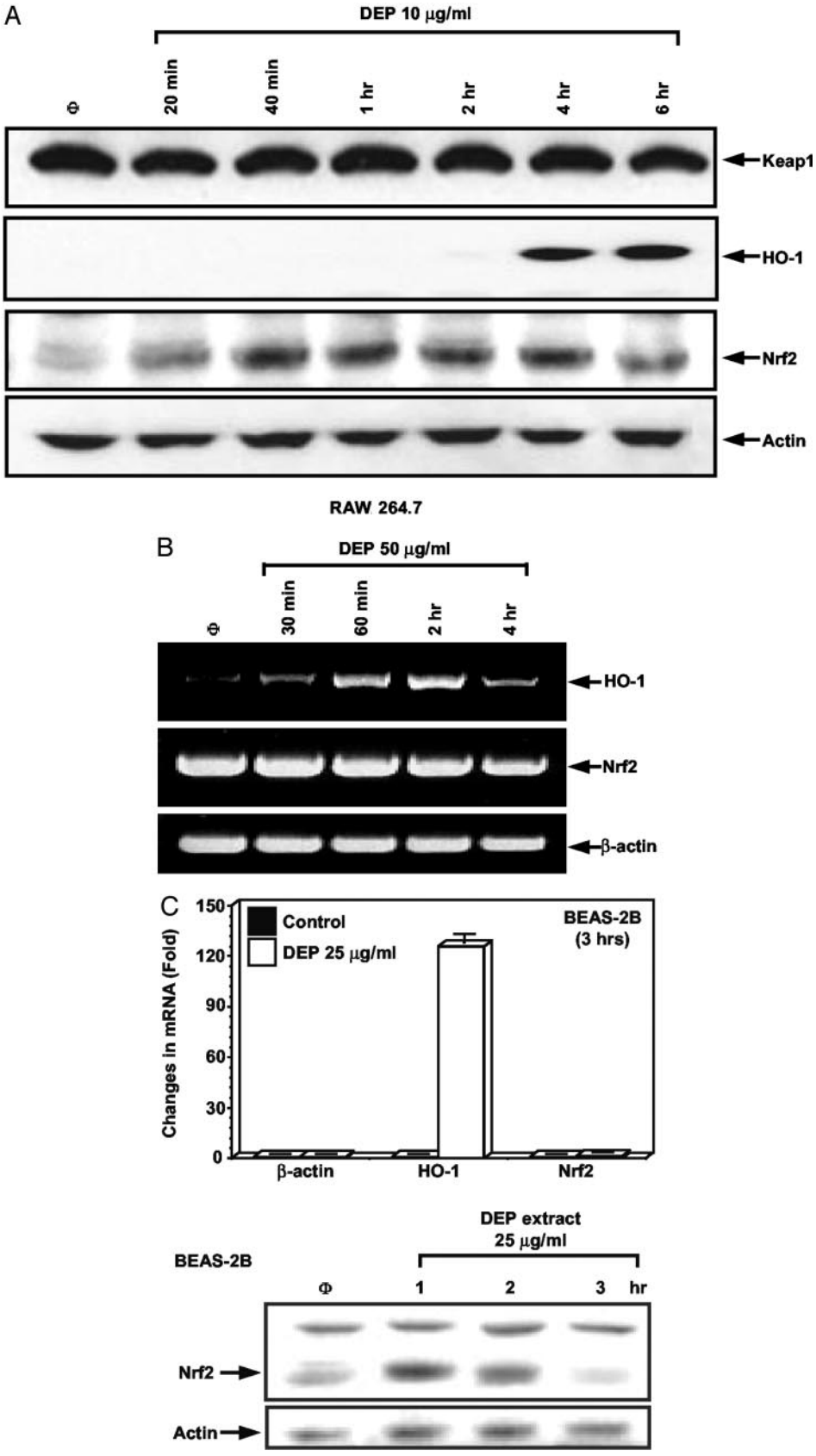

FIGURE 6. Nrf2 protein expression is regulated at a post-transcriptional level. *A*, Time-dependent increase in HO-1 and Nrf2 protein in whole cell lysates prepared from RAW 264.7. Cells were treated with 10 μg/ml crude DEP extract for the indicated time periods. Whole cell lysates were prepared and blotted with polyclonal anti-Keap1, anti-Nrf2, or monoclonal antio-HO-1 Ab. B, Differential HO-1 and Nrf2 mRNA expression in RAW 264.7. Cells were treated with 50 μg/ml crude DEP extract for the indicated time period. RT-PCR was performed as described in *Materials and Methods*. β-Actin was used as the internal control. *C*, Quantitative analysis of HO-1 and Nrf2 gene expression in BEAS-2B. Cells were exposed to 25 μg/ml crude DEP extract for 3 h before real-time PCR analysis. Immunoblot to show the increase in Nrf2 protein by DEP extract in BEAS-2B cells.

electrophilic and/or pro-oxidative mechanisms interfere in the proteasomal degradation of Nrf2. This results in the nuclear accumulation of this transcription factor. Ambient ultrafine particles, which have a higher organic carbon content than PM_{10}, were more potent than coarse particles in their ability to induce ARE activation. These data are of considerable importance in understanding susceptibility to PM-induced adverse health effects in the lung, including asthma exacerbation.

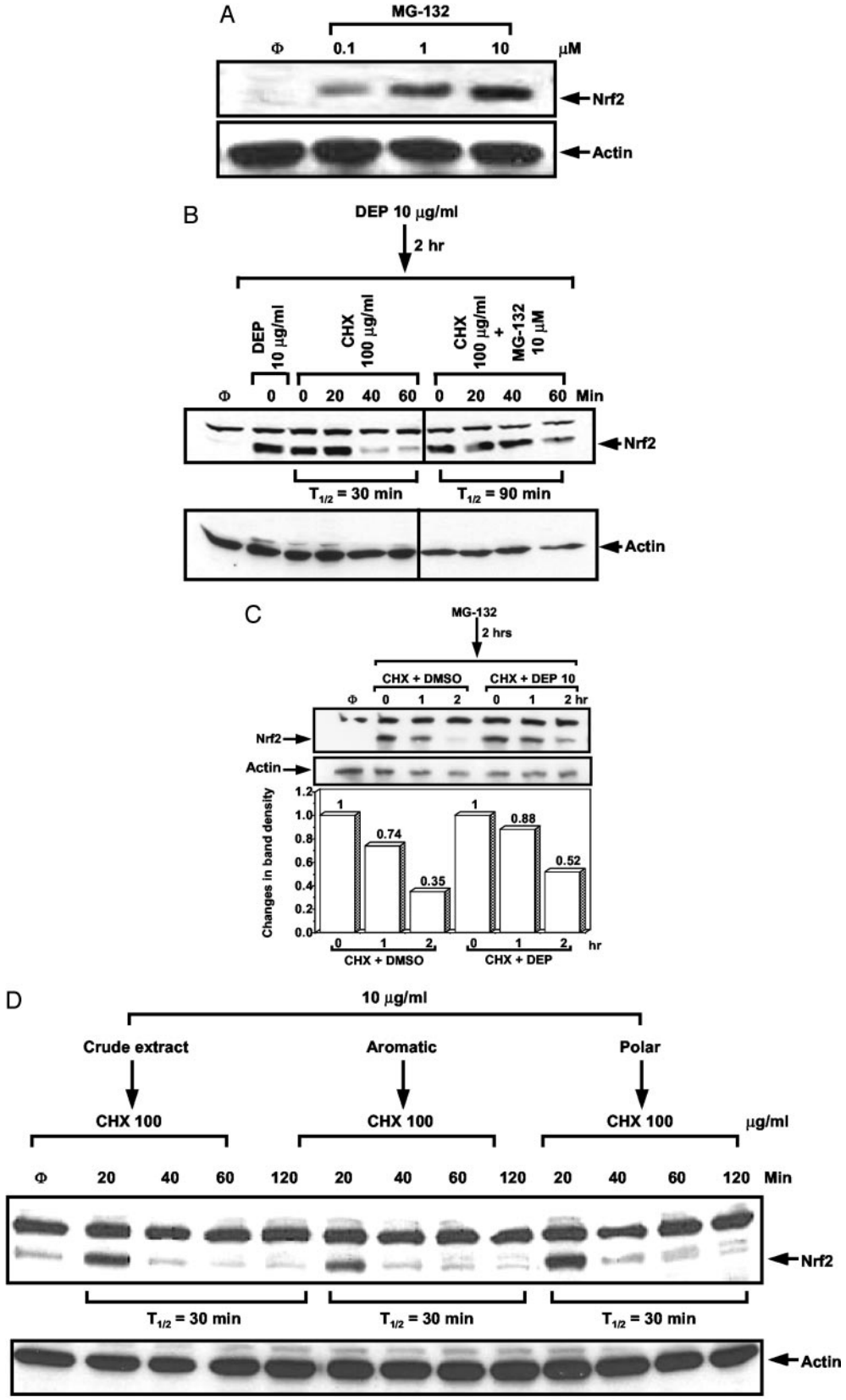

FIGURE 7. DEP chemicals induce Nrf2 protein accumulation by inhibiting its proteasomal degradation. *A*, Dose-dependent Nrf2 protein accumulation by MG-132. RAW 264.7 cells were treated with this 26S proteosome inhibitor for 2 h. Whole cell lysates were prepared and probed with polyclonal anti-Nrf2 Ab. Protein half-life was calculated by plotting protein band density vs time. *B*, Pulse-chased experiment showing Nr2 expression in the presence of CHX. RAW 264.7 cells were exposed to 10 μg/ml crude DEP extract for 2 h in low serum DMEM. The cells were washed, and fresh medium containing CHX (100 μg/ml) and either DMSO or 10 μM MG-132 was added for the indicated time period. *C*, DEP enhances Nrf2 stability if combined with CHX. RAW 264.7 cells were stimulated with 10 μM MG-132 for 2 h. After washing, fresh medium containing CHX (100 μg/ml) and either DMSO or 10 μg/ml DEP extract was added for the indicated time periods. *D*, Nrf2 protein stability is increased by the aromatic and polar fractions. Cells were treated with 10 μg/ml aromatic or polar fractions for 2 h, followed by washing and addition of CHX as described in *A*.

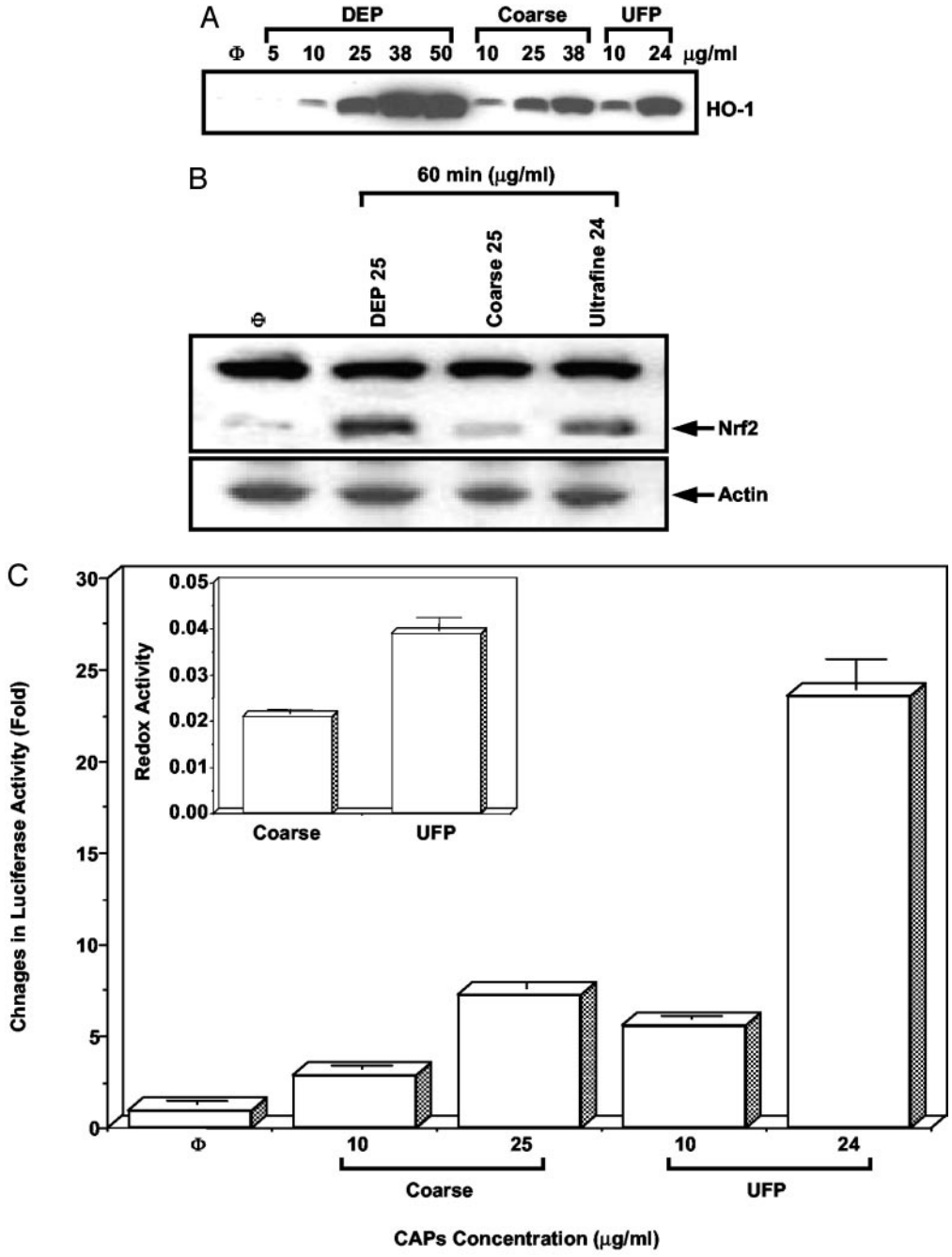

FIGURE 8. Concentrated ambient particles induce HO-1 and Nrf2 accumulation. *A*, HO-1 immunoblot after RAW 264.7 cells were exposed to different particle types for 16 h at the indicated concentrations. Intact DEP were used as a positive control. *B*, Nrf2 immunoblot on whole cell lysates after exposure to the different particle types. *C*, ARE-luciferase activity correlated with the in vitro redox activity of the particles. ARE-luciferase activity was measured after stable transfected RAW 264.7 cells were exposed to concentrated ambient particles for 6 h. Values represent two separate wells with duplicate readings per well. *Inset*, In vitro redox activity was analyzed using DTT assay as described in *Materials and Methods*. Redox activity was expressed as nanomoles of DTT per minute per microgram. UFP, ultrafine particles.

To neutralize the pro-oxidative effects of xenobiotics, higher animals have developed a finely coordinated battery of genes that encode phase II and antioxidant enzyme expression. This includes the expression of various SOD isoforms, catalase, glutathione peroxidase, glutathione reductase, various GST isoforms, NQO1, and HO-1 (20). A number of these enzymes play a role in defending against the injurious effects of electrophiles and oxidative chemicals (11, 25, 26). HO-1, for instance, is a key antioxidant enzyme that protects against the cytotoxic effects of pro-oxidative DEP chemicals and ROS (11, 26). NQO1 performs two-electron reductions that assist in the detoxification of quinones, whereas the GSTs exert a variety of effects, including ROS removal and detoxification of oxy-PAH components such as quinones (11, 57). It is likely, therefore, that this oxidant stress and electrophile response system plays a key role in defending against toxic air pollutants. This idea is substantiated by increased DNA adduct formation in the lungs of Nrf2 knockout mice exposed to diesel exhaust fumes (58). Moreover, substantiating data in humans show that a GSTM1 (null) genotype leads to increased allergic airway responsiveness during allergen challenge (59). It is possible, therefore, that altered or polymorphic expression of phase II enzymes may

Table VI. *Elemental and organic carbon content in CAPs collected in Los Angeles basin*[a]

	Coarse, $\mu g/m^3$ (%)	Ultrafine, $\mu g/m^3$ (%)
OC	1.786 (17.4)	6.911 (79.9)
EC	0.817 (7.9)	0.844 (9.8)
Nitrate	2.25 (21.9)	0 (0)
Sulfate	0.972 (9.5)	0.381 (4.4)
Metals and elements	5.638 (54.9)	1.689 (19.5)

[a] CAPs were collected in Downey, CA, in May 2003. The amounts of OC, EC, nitrate, sulfate, metals, and elements were analyzed as described in *Materials and Methods*.

determine which human subjects are more prone to developing asthma during PM exposure.

The coordinated responses of phase II genes are regulated through a *cis* element, known as the ARE or the electrophile response element (11). Nrf2 in association with small Maf proteins (e.g., Maf G) as well as AP-1 transcription factors play key roles in the activation of the ARE (30–33). The importance of Nrf2 is illustrated by the failure to induce phase II enzyme expression in the lungs and livers of Nrf2-deficient mice, thereby rendering these animals more susceptible to the effects of hyperoxia, pro-oxidative food chemicals, and drugs (e.g., acetaminophen) (45, 58, 60, 61). Our results using peritoneal macrophages from *Nrf2*$^{-/-}$ mice also provided direct evidence for the role of Nrf2 in regulating HO-1 expression in response to pro-oxidative DEP chemicals (Fig. 3*B*). This is compatible with interference in phase II enzyme expression under conditions of hyperoxic lung injury (25). The ability of Nrf2 to transcriptionally activate these genes is regulated in part by a cytoplasmic protein, Keap1 (34–38). Keap1 contains a C-terminal Kelch domain that interacts directly with an Nrf2 regulatory site, known as the Neh2 domain (36, 62). However, the molecular explanation for the cytoplasmic retention of Nrf2 is poorly understood. Several models have been proposed to explain the escape of Nrf2 from Keap1-mediated repression (34, 36, 38, 56). One suggestion is that electrophilic chemicals, such as the quinones, covalently modify one or more of the 27 thiol groups present in Keap1 (56). Zhang et al. (56) identified two critical Keap1 cysteine residues, C273 and C288, that are required for Nrf2 ubiquitination. This ubiquitination event is probably responsible for the rapid proteasomal degradation and short half-life of Nrf2 under homeostatic conditions (34–37). However, the ability of these cysteines to induce Nrf2 ubiquitination may be disrupted in the presence of electrophilic compounds, allowing Nrf2 to escape Keap1-dependent degradation (56). This could explain the increased Nrf2 half-life by DEP chemicals (Fig. 7*C*). The thiol hypothesis also explains why NAC interferes with Nrf2 accumulation and activation of the ARE in our system (Figs. 5*B* and 2*C*). NAC has a number of actions that may explain this effect. In addition to its ROS scavenging effects and action as a GSH precursor, NAC uses its Src homology group to participate in electrophilic interactions. This may involve direct binding to electrophilic DEP chemicals (24).

However, whereas thiol modification may explain why aromatic and polar chemical compounds increase Nrf2 protein accumulation (Fig. 5*A*), this does not explain Nrf2 release from Keap1. Additional hypotheses have been proposed to explain this effect. One is the phosphorylation of Nrf2 by protein kinase C (PKC) (38). PKC phosphorylates a Nrf2 S40 residue and induces its in vitro dissociation from Keap1 (38). However, phosphorylation of this residue has not been demonstrated in vivo, and the S40A-Nrf2 mutant behaved identically with the wild-type protein in its interaction with Keap1 (38). We could not demonstrate interference in the activation of the ARE by PKC inhibitors in our system (not shown).

A third possibility, which is not mutually exclusive of the roles of C273 and C288, is that DEP chemicals may target additional cysteines on Keap1 or Nrf2. One example is the Keap1 residue, C151; this cysteine plays a unique role in the response of this pathway to oxidative stress (56). One possibility is that C151 acts as a redox-sensitive switch that is targeted by electrophilic DEP compounds or by ROS that is generated as an independent event. Quinones are functionalized polar chemicals that induce $O_2^{\cdot-}$ generation when added to lung microsomes (17). This redox cycling activity is catalyzed by NADPH-P450 reductase (17, 18) and could explain why the polar is more active than the aromatic fraction in inducing Nrf2 accumulation and the activation of the ARE (Figs. 2*A* and 5*B*). This idea is also in keeping with the increased potency of the polar fraction in the DTT assay (Fig. 2*D*). This assay is a measure of the in vitro redox cycling capacity of the oxy-PAH compounds present in ambient PM and DEP (7, 42). It is interesting, therefore, that ultrafine particulates, which have a higher in vitro redox cycling capability than coarse particles (7), are more potent inducers of HO-1 expression, Nrf2 accumulation, and activation of the ARE (Fig. 8). A possible way to explain the lesser effect of the aromatic fraction is that the PAHs need to be converted to quinones, e.g., by cytochrome P450 1A1, before they become functionalized (63, 64).

It is important to point out that the release of Nrf2 from Keap1 may not be an essential regulatory step in the Nrf2/ARE pathway. This idea is derived from the fact that because Nrf2 bound by Keap1 is targeted for proteasomal degradation, it does not accumulate to significant levels in the cytoplasm of unstimulated cells (34, 36). Furthermore, Nrf2 that accumulates to high levels in the nucleus of stimulated cells represents primarily, if not exclusively, de novo synthesized protein (34, 36). Given these observations, it is possible that oxidants promote nuclear accumulation of Nrf2 not by affecting the release of Nrf2, which in any case would represent a minimal amount of Nrf2, but by preventing newly synthesized Nrf2 from associating with Keap1. In the absence of such association, Nrf2 will escape proteasomal degradation and by default be directed to the nucleus. Interestingly, and consistent with this hypothesis, Zhang and Hannink (56) have observed minimal release of Nrf2 from Keap1 in response to electrophiles. Although molecular details about the roles of Nrf2 and Keap1 in phase II enzyme expression is still unclear, proteasome-mediated Nrf2 degradation appears to be an essential step in both mouse and human cells (65–67).

Oxidative stress may be the central mechanism by which ambient PM induce adverse health effects. We have recently demonstrated that PM-induced oxidative stress is a multi-tier response, in which cytoprotective responses transition to injurious effects as the level of oxidative stress increases (11, 16). The induction of Nrf2-mediated phase II enzyme expression is an integral component of the cytoprotective response, which is triggered at the lowest level (tier 1) of oxidative stress. This system is designed to prevent further oxidative stress, which may escalate into inflammation and cytotoxicity (11, 30–33). In this role, phase II enzymes may be particularly important in averting allergen sensitization and allergic airway inflammation in atopic people (1, 11). In this regard, it is well known that DEP and ambient PM act as adjuvants that promote TH2 immune deviation during cochallenge with an allergen (1, 11). Moreover, several animal and human studies show that oxidative stress is important in asthma, and that antioxidant defense pathways are important in the prevention of disease progression (1, 11).

There is growing evidence that genes involved in xenobiotic detoxification and antioxidant defense could serve as susceptible genes for asthma pathogenesis (59, 68–70). For instance, individuals who are homozygous for the GSTM1 (null) genotype, have been shown to have an increased risk for asthma development or allergic nasal responses (59). In contrast, homozygous GSTP1 expression confers a protective effect on asthma and has also been shown to protect against toluene di-isocyanate-induced asthma in spray painters (68). Although we still lack evidence for the involvement of Nrf2, it has recently been demonstrated that the HO-1 promoter expresses a polymorphism that defines gene inducibility under oxidative stress conditions (70). This polymorphism impacts emphysema development in Japanese male smokers (70). We suggest that related polymorphisms in phase II enzymes determine their Nrf2 responses and could determine PM susceptibility. Although we are still uncertain about the cellular impact of oxidative stress and antioxidant defense on Th_2 pathways, we are exploring the possibility that the DEP adjuvant effect is mediated through oxidative stress effects in the lung.

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