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Ambient Vapor Samples Activate the Nrf2-ARE Pathway in Human Bronchial Epithelial BEAS-2B Cells

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ABSTRACT: Ambient air pollutants have been reported to induce oxidative stress based inflammatory responses in humans and experimental animals. However, most of these reports describe the actions of the particulate phase of ambient and exhaust samples. We describe here results of studies investigating the actions of the vapor phase of ambient air samples collected in the midtown area of Los Angeles on human bronchial epithelial BEAS-2B cells using DNA microarray analysis. Among 26 genes whose expression increased fourfold or more, four genes were associated with detoxifying genes regulated by the transcription factor Nrf2. Consistent with these results, the vapor samples activate the Nrf2-ARE pathway, resulting in up-regulation of heme oxygenase-1 (HO-1), glutamate cysteine ligase modifier subunit, and cystine transporter (xCT) mRNA and proteins. No appreciable increases in pro-inflammatory genes were observed. These results suggest that ambient vapor samples activate the Nrf2-ARE pathway but not an inflammatory response. Also, treatment of the vapor samples with glutathione resulted in reduction in the Nrf2 activation and HO-1 induction, suggesting that electrophiles in vapor samples contribute to this Nrf2-dependent antioxidant or adaptive response. Environ Toxicol 00: 000–000, 2013.
Keywords: ambient air sample; vapor phase; electrophile; microarray; Nrf2

INTRODUCTION

It is well recognized that exposure to air pollutants can cause the exacerbation of asthma and cardiovascular diseases (Ebtekar, 2006; Brook et al., 2010; Franchini and Mannucci, 2012) and inflammation-related processes are known to play a key role in these effects (Murdoch and Lloyd, 2010; Van Eeden et al., 2012). Consistent with this notion, air pollution particles have been shown to activate Nf-κB and up-regulate pro-inflammatory genes such as eotaxin and interleukins (Quay et al., 1998; Takizawa et al., 2003; Becker et al., 2005).

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Ambient air pollutants contain chemicals present in both the vapor- and particle-phases, but most health assessment studies have focused on ambient and exhaust particles. In recent studies of ambient vapor- and particle phase samples collected simultaneously in Riverside (CA), we found that redox active compounds referred to as prooxidants were mainly associated with the particle-phase, whereas electrophiles were mostly found in the vapor-phase (Eiguren-Fernandez et al., 2010). Electrophiles can activate different cell signaling pathways by covalent modification of key regulatory proteins (Jacobs and Marnett, 2010; Rudolph and Freeman, 2009), and we have found that upon exposure to these vapor-phase samples, mouse macrophage Raw264.7 cells responded by activating NF-E2–related factor 2 (Nrf2), a transcription factor involved in cellular protection against both oxidative and electrophilic chemical insults, via covalent modification of Kelch-like ECH-associated protein 1 (Keap1) in the absence of measureable reactive oxygen species (ROS) production (Iwamoto et al., 2010). Although Nrf2 is known to affect downstream genes regulating antioxidants and detoxifying proteins, genome-wide alterations of the expression of Nrf2-dependent and -independent genes caused by vapor samples exposure has not been clarified. To address this issue, we first performed DNA microarray analysis of human bronchial epithelial BEAS-2B cells exposed to ambient vapor samples to determine whether they could activate inflammatory as well as protective responses. We then examined the activation of the Nrf2-antioxidant response element (ARE) pathway by vapor samples and the contribution of electrophiles. The results showed no evidence for contributions to inflammation by components of the vapor phase.

MATERIALS AND METHODS

Materials

Dithiothreitol (DTT), 5,5′-dithiobisbis-(2-dinitro)benzoic acid (DTNB), diethylenetriaminepentaacetic acid (DTPA), and rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Sigma Chemical Co (St Louis MO). Anti-Nrf2 antibody (sc-13032), anti-glutamate cysteine ligase modifier subunit (GCLM) antibody (sc-22754), and anti-GAPDH antibody (sc-20357) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HO-1 antibody (SPA-895) was purchased from Stressgen (Victoria, Canada). Anti-cystine transporter (xCT) antibody (ab37185) was obtained from Abcam (Cambridge, MA). Horseradish peroxidase (HRP)–conjugated anti-rabbit IgG secondary antibodies was obtained from Cell Signaling Technology (Beverly, MA). All other reagents used were of the highest purity available.

Vapor-Phase and Particulate Matter Sample Collection and Extraction

Sample collection and extraction were conducted as reported previously (Eiguren-Fernandez et al., 2008, 2010). Vapor-phase and particulate matter samples were collected the mid town area of Los Angeles near the University of Southern California (USC) using PM2.5 medium-vol samplers (Tisch Environmental Model 1q202 Cleves OH) during June (from 17th to 22th) and July (from 1st to 7th) in 2008. The particles were collected on Teflon coated glass fiber filters (Pall Corporation, East Hills, NY) and the corresponding vapors in 20 g of XAD-4 resin (Acros, Thermo Frisher Scientific) beds. The XAD resins containing the volatile organic species were extracted with dichloromethane (100 mL) by sonication for 30 min at room temperature. The dichloromethane was then removed under reduced pressure and the residue reconstituted in dimethyl sulfoxide (DMSO) to a final concentration of $\sim$300 m^3/mL. Punches of the Teflon coated filters containing PM2.5 samples were extracted by sonication in distilled water and the resulting aqueous suspension subjected to analysis.

DTT Assay

This procedure measures the prooxidant content of the sample based on its ability to transfer electrons from DTT to oxygen (Kumagai et al., 2002; Cho et al., 2005). Thus, aliquots of vapor-phase and particulate matter samples were incubated with DTT for times varying from 10 to 30 min in aqueous buffer. The reaction was quenched at specific times and after addition of DTNB to complex with the remaining DTT, the absorption at 412 nm measured. The rates are calculated by averaging duplicate runs, and are blank corrected. Since DTT can be oxidized by high concentrations of metal ions (Netto and Stadtman, 1996), the contribution of metals to the DTT-based redox activity was also determined by adding the metal chelator DTPA (20 μM) to one set of the samples. The units used are the nanomoles of DTT consumed per minute per m^3 of air sample.

GAPDH Assay

This assay measures the electrophile content in the sample, from their ability to inhibit or inactivate the thioate enzyme GAPDH through covalent bonding (Shinyashiki et al., 2008). In brief, a mixture of 1 unit of rabbit GAPDH was incubated with aliquots of the organic extracts of vapors and particles or water suspension under argon gas at 25°C for 120 min. At this time point the reaction was quenched by adding an equal volume of cold DTT solution, and GAPDH activity, measured as the rate of nicotinamide adenine dinucleotide (NADH) formation, was monitored by its absorption at 340 nm. The ability to inactivate the enzyme is expressed as the

TABLE I. Human Gene-Specific Primers for Quantitative Real-Time RT-PCR

Gene	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	Product Size (bp)
HO-1	GCCACCAAGTTCAAGCAGCTCTA	AGCAGCTCCTGCAACTCCTCAA	139
xCT	ATGTCCGCAAGCACACTCCTCTA	TCGAAGATAAATCAGCCCAGCAA	158
GCLM	CCAGATGTCTTGGAATGCACTGTA	GCTGTGCAACTCCAAGGACTGA	166
GAPDH	GCACCGTCAAGGCTGAGAAC	GTGGTGAAGACGCCAGTGGA	139

equivalents of *N*-ethylmaleimide (NEM), the standard electrophile. Samples were run in triplicate and values reported as averages. Although the assay is limited to those structures capable of interacting with the catalytic center of the enzyme, it provides a quantitative measure of the electrophilic content which can be used in comparison studies. The units used are the equivalents of *N*-ethylmaleimide per m^3.

Cells and Cell Culture

Human bronchial epithelial cell line BEAS-2B cells (ATCC, Manassas, VA) were cultured in a humidified atmosphere of 5% CO_2 at 37°C using bronchial epithelial cell growth medium (BEGM; Lonza) consisting of bronchial epithelial basal medium (BEBM; Lonza) with growth factor supplements with human recombinant epidermal growth factor, bovine pituitary extract, hydrocortisone, insulin, transferrin, epinephrine, retinoic acid, and triiodothyronine. In each experiment, BEAS-2B cells were seeded at a cell density of 2×10^4 cells/cm^2 and cultured for 48 h in BEGM. Before treatment, cells were cultured in BEBM (serum-free medium) overnight and then treated with each chemical in BEBM.

Microarray Analysis

Total RNA was extracted and purified from the exposed cells (6 h) using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Overall changes in gene expression were evaluated using two-color microarray-based gene expression analysis as previously described (Toyama et al., 2011). Briefly, the Cy3- and Cy5-labelled cRNA mixture was fragmented at 60°C for 30 min in a solution containing 1 × Agilent fragmentation buffer and 2 × Agilent blocking agent. After washing, hybridized slides were scanned with an Agilent DNA Microarray Scanner (G2565CA) using the two-color scan setting for 8 × 60k array slides (scan area: 61.0 × 21.6 mm; scan resolution: 3 mm; dye channel set to green and red; photomultiplier tube set to 100%).

Real-Time PCR

After treatment with vapor samples for 6 h, total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA), and cDNA was synthesized from the mRNA using the High Capacity RNA-to-cDNA™ Kit (Applied Biosystems, Foster, CA). Real-time polymerase chain reaction (PCR) was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) with 0.6 μg cDNA and 0.2 μM primers (Table I) on a 7500 Real Time PCR system (Applied Biosystems). Thermal cycling parameters were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Melting curve analysis and agarose gel electrophoresis with ethidium bromide staining was conducted to ensure a single PCR product of correct amplicon length. Levels of HO-1, GCLM, xCT, and GAPDH mRNA in each RNA sample were quantified by the relative standard curve method. Fold-change for each gene was assessed after normalization of the intensity value to GAPDH.

Western Blotting

After treatment with vapor samples for 1, 3, 6, 12, or 24 h, cells were washed twice with ice-cold PBS. Total cell proteins were prepared by lysis in SDS sample buffer (50 mM Tris-HCl, pH 6.8; 2% SDS; and 10% glycerol), followed by incubation at 95°C for 10 min. A crude membrane fraction was prepared by differential centrifugation, as previously described (Shinkai et al., 2009). We determined protein concentration using a BCA protein assay reagent kit (Pierce) before 2-mercaptoethanol and bromophenol blue was added to each sample. The cellular proteins were separated by SDS-polyacrylamide gel electrophoresis on a polyacrylamide gel and electrotransferred onto a polyvinyl difluoride membrane (Bio-Rad, Hercules, CA) at 2 mA/cm^2 for 1 h according to the method of Kyhse-Andersen (1984).

TABLE II. Redox and Electrophilic Activity in the Vapor and Particulate Matter Samples

Sample	Prooxidant Content (DTT Assay Activity/m^3)	Electrophile Content (NEM Equivalents/m^3)
VP1	0.185 (0.151–0.214)	1.192 ± 0.078
VP2	0.103 (0.092–0.132)	0.572 ± 0.018
PM1	0.660 (0.551–0.770)	0.058 ± 0.0059
PM2	0.937 (0.864–1.01)	0.098 ± 0.0018

Prooxidant content and electrophile content in the vapor and particle matter samples were measured, respectively, as described in the Materials and Methods section. DTT results are obtained as the best fit of 4 duplicate time vs. consumption data points to a straight line, with 95% confidence values shown in parentheses. The GAPDH assay results represent the mean ± SE of three or more independent analyses.

TABLE III. Increased Gene Expression in BEAS-2B Cells Exposed to VP1

	Gene Name	Gene Symbol	Accession Number	Fold Change
1	Heme oxygenase 1	HO-1	NM_002133	18.0
2	Solute carrier family 7, member 11	SLC7A11	NM_014331	16.7
3	HtrA serine peptidase 3	HTRA3	NM_053044	15.2
4	EP300 interacting inhibitor of differentiation 3	EID3	NM_001008394	9.6
5	Aldo-keto reductase family 1, member B10	AKR1B10	NM_020299	8.2
6	Hexokinase domain containing 1	HKDC1	NM_025130	8.1
7	Heparan sulfate 3-O-sulfotransferase 2	HS3ST2	NM_006043	7.6
8	CMT1A duplicated region transcript 1	CDRT1	NM_006382	7.1
9	Adiponectin	ADIPOQ	NM_004797	6.3
10	Potassium voltage-gated channel, Isk-related family, member 2	KCNE2	NM_172201	6.2
11	1-Acylglycerol-3-phosphate O-acyltransferase 9	AGPAT9	NM_032717	5.9
12	Rho guanine nucleotide exchange factor (GEF) 7	ARHGEF7	NM_145735	5.8
13	Glutamate-cysteine ligase, modifier subunit	GCLM	NM_002061	5.7
14	Rho GTPase activating protein 25	ARHGAP25	NM_001007231	5.7
15	Heat shock 70kDa protein 6	HSPA6	NM_002155	5.6
16	Chromosome 3 open reading frame 35	C3orf35	NM_178339	5.5
17	Zinc finger protein 469	ZNF469	NM_001127464	5.2
18	cAMP responsive element binding protein 5	CREB5	NM_182898	4.7
19	MORN repeat containing 5	MORN5	NM_198469	4.7
20	Oxidative stress induced growth inhibitor 1	OSGIN1	NM_013370	4.6
21	Distal-less homeobox 2 (DLX2)	DLX2	NM_004405	4.4
22	BCL2-like 10 (apoptosis facilitator)	BCL2L10	NM_020396	4.3
23	Integrin, alpha 4	ITGA4	NM_000885	4.3
24	Macrophage expressed 1	MPEG1	NM_001039396	4.3
25	Solute carrier family 5 (sodium/glucose cotransporter), member 1	SLC5A1	NM_000343	4.2
26	Tribbles homolog 1 (Drosophila)	TRIB1	NM_025195	4.0

BEAS-2B cells were exposed to VP1 (2.68 m^3/mL) for 6 h and changes in gene expression were assessed by DNA microarray. Genes whose expression increased fourfold or more are listed.

Membranes were blocked with 5% skim milk in (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; and 0.1% Tween 20) and then incubated with primary antibodies for 1 h at room temperature. The membranes were washed and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (Chemi-Lumi One L; nacalai) and scanned by LAS 3000 (Fujifilm, Tokyo, Japan). The bands were quantified using ImageJ software. Representative blots are shown from at least two independent experiments.

Luciferase Assay

DNA transfections were performed by using FuGENE HD transfection reagent (Roche Applied Sciences, Indianapolis, IN) according to the manufacturer's instructions. Briefly, cells were cultured in 24-well. ARE-luciferase cDNA (0.5 μg/well) and pRL-TK cDNA (0.05 μg/well) or transfection reagent (1 μg/well) were mixed with serum-free media. Before addition to the cells, the DNA solution and transfection reagent solution were mixed together and incubated for 15 min at room temperature to allow the formation of complexes. The complexes were mixed with the culture media and incubated for 24 hr to transfect. After transfection, the cells were treated with VP1 or VP2 for 6 or 12 h and then luciferase activity was measured in cellular extracts according to the manufacturer's instructions (Dual-Luciferase reporter assay system; Promega, WI, USA) with a lumiometer (Promega).

Statistical Analysis

Statistical analysis was performed using Microsoft Excel (Microsoft, Redmond, WA). Statistical significance was assessed with Student's *t*-test, and $p < 0.05$ was considered significant. Linear regression analyses of DTT assay results were performed with Prism (GraphPad Software, San Diego CA).

RESULTS

Chemical Properties of Vapor Samples

In characterizing the chemical properties of the complex mixtures of air pollutants, we have developed assay procedures that characterize two important chemical reactivities, the ability to generate reactive oxygen species in the presence of electron donors, or prooxidant activity, and the ability to form covalent bonds with nucleophilic functions on proteins, or

TABLE IV. Decreased Gene Expression in BEAS-2B Cells Exposed to VP1

	Gene Name	Gene Symbol	Accession Number	Fold Change
1	Tripartite motif-containing 9	TRIM9	NM_052978	0.14
2	Germinal center expressed transcript 2	GCET2	NM_001008756	0.18
3	Formyl peptide receptor 1	FPR1	NM_002029	0.23
4	Major intrinsic protein of lens fiber	MIP	NM_012064	0.26
5	Selenium binding protein 1	SELENBP1	NM_003944	0.28
6	Usher syndrome 1G (autosomal recessive)	USH1G	NM_173477	0.28
7	Palmdelphin	PALMD	NM_017734	0.30
8	Kazal-type serine peptidase inhibitor domain 1	KAZALD1	NM_030929	0.30
9	Mitogen-activated protein kinase kinase 6	MAP2K6	NM_002758	0.30
10	Coiled-coil domain containing 102B	CCDC102B	NM_024781	0.31
11	Lysosomal protein transmembrane 5	LAPTM5	NM_006762	0.34
12	Tumor protein p73	TP73	NM_005427	0.34
13	v-Myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	MYCL1	NM_005376	0.35
14	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	PPARGC1A	NM_013261	0.35
15	Zinc finger protein 683	ZNF683	NM_173574	0.35
16	Steroid sulfatase (microsomal), isozyme S	STS	NM_000351	0.35
17	Podocalyxin-like 2	PODXL2	NM_015720	0.36
18	RAB9B, member RAS oncogene family	RAB9B	NM_016370	0.36
19	PR domain containing 5	PRDM5	NM_018699	0.37
20	HLA complex P5	HCP5	NM_006674	0.38
21	Tumor necrosis factor, alpha-induced protein 8-like 3	TNFAIP8L3	NM_207381	0.39
22	MORN repeat containing 3	MORN3	NM_173855	0.39
23	Wingless-type MMTV integration site family, member 4	WNT4	NM_030761	0.39
24	CXXC finger 4	CXXC4	NM_025212	0.39
25	Tumor necrosis factor (TNF superfamily, member 2)	TNF	NM_000594	0.40

BEAS-2B cells were exposed to VP1 (2.68 m^3/mL) for 6 h and changes in gene expression were assessed by DNA microarray. Genes whose expression decreased less than 0.4-fold or more are listed.

electrophilic activity. We have chosen to chemically characterize the samples in this manner because analyses for individual substances would require large quantities of sample and an arbitrary selection of analytes that may or may not affect cells. Instead, we hope to characterize in quantitative terms, the ability of samples to carry out two reactions known to affect biological systems. Prooxidant content is measured by the ability of the sample to transfer electrons from DTT to oxygen (Li et al., 2003; Cho et al., 2005) and electrophile content is measured by the ability of the sample to inactivate the thiol enzyme, GAPDH (Shinyashiki et al., 2008, 2009). The results of these assays, performed on the particle and vapor phases of the samples indicated that 70–80% of the prooxidants were found in the particle phase and most (85–95%) of the electrophiles were found in the vapor phase (Table II). Since the majority of the electrophiles were in the vapor phase the cellular responses to this phase were the focus of the study.

Global Gene Analysis by Microarray

Exposure of BEAS-2B cells to VP1 at 2.68 m^3/mL for 6 h did not cause cell death (data not shown). Under these conditions, VP1 increased the expression of 461 genes more than twofold and decreased the expression of 180 genes less than 0.5-fold. The expression of 26 genes increased more than fourfold (Table III), and the expression of 25 genes decreased up to 0.4-fold (Table IV) in response to VP1. Among the genes that are regulated by Nrf2, expression of HO-1, SLC7A11 (xCT), AKR1B10, and GCLM, was markedly increased. There was also a somewhat smaller increase in the expression of the Nrf2-regulated genes TXNRD1 (thioredoxin reductase; 3.7-fold), SRXN1 (sulfredoxin 1; 3.3-fold), NQO1 (NAD(P)H quinone oxidoreductase 1; 2.4-fold) and GCLC (glutamate-cysteine ligase, catalytic subunit; 2.4-fold) during VP1 exposure (data not shown). It is noteworthy that VP1 did not induce inflammatory responsive genes such as interleukins, tumor necrosis factor α (TNFα), cyclooxygenase-2 (COX-2), but VP1 decreased TNF expression (0.4-fold) at these concentrations. It should be noted that there is possibility that late-response genes are not detected because we only conducted this analysis after 6 h exposure.

Vapor Samples Activate Nrf2-ARE Pathway

To confirm the microarray results, we performed RT-PCR. As shown in Figure 1, exposure of BEAS-2B cells to VP1

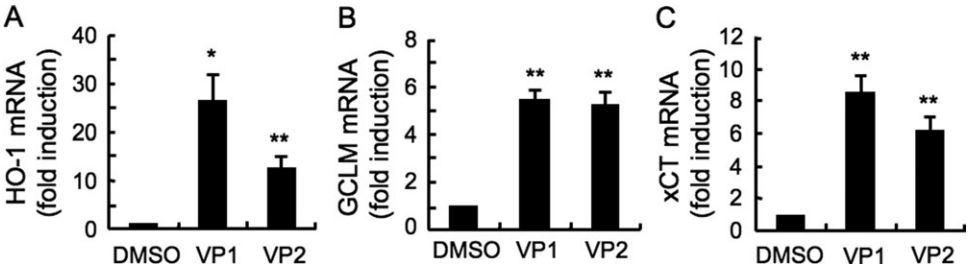

Fig. 1. Up-regulation of HO-1, GCLM, xCT mRNA levels by vapor phase samples in BEAS-2B cells. Cells were exposed to VP1 (2.68 m^3/mL) or VP2 (3.19 m^3/mL) for 6 h. HO-1 (A), GCLC (B), and xCT (C) mRNA levels were determined by real-tme PCR. All mRNA expression levels were normalized to GAPDH mRNA levels and expressed as fold induction relative to control cells. Each value represents the mean ± SE of three independent experiments performed in duplicate. *$p < 0.05$ and **$p < 0.01$ compared with DMSO.

or VP2 resulted in a marked increase in HO-1, GCLM, and xCT mRNA expression. Consistent with these results, VP1 and VP2 significantly induced ARE-driven luciferase activity (Fig. 2). Furthermore, immunoblot analyses showed that the samples increased levels of activated Nrf2, resulting in the up-regulation of HO-1, GCLM and xCT proteins (Fig. 3).

Involvement of Electrophiles in the Activation of Nrf2 by Vapor Samples

To investigate the contribution of electrophiles to vapor sample-mediated Nrf2 activation, BEAS-2B cells were pre-incubated with glutathione (GSH, 10 mM) for 1 h then exposed to either VP1 or VP2 at the indicated concentrations. As expected, treatment of the vapor samples with the nucleophilic GSH significantly suppressed the Nrf2 activation and HO-1 induction (Fig. 4).

DISCUSSION

This study shows that ambient vapor samples collected from midtown Los Angeles activate the Nrf2-ARE pathway without inducing inflammatory genes in BEAS-2B cells. Based on the DNA microarray results, the Nrf2-ARE pathway is the primary response to ambient vapor samples, suggesting that the air pollutant–induced inflammatory response may be due to particle components. In fact, several researchers have reported that particles induce pro-inflammatory genes (Takizawa et al., 2003; Becker et al., 2005; Hasegawa et al., 2011). We have reported earlier that ambient vapor samples collected from Riverside (CA) also activate Nrf2, resulting in the up-regulation of HO-1, GCL, GSH *S*-transferase proteins in Raw264.7 cells (Iwamoto et al., 2010), indicating that the ability of ambient vapor samples to activate Nrf2 may not be region-specific. Furthermore, the up-regulation was observed under conditions of minimal oxidative stress, suggesting that the vapor phase effects are likely due to electrophile action.

HO-1 is the most up-regulated gene by VP1 and can protect against oxidative stress and apoptotic cell death (Gozzelino et al., 2010); it has also been shown to have anti-inflammatory properties (Paine et al., 2010). It is thus a protective protein and since the inflammation-associated gene, TNF, is down-regulated gene by VP1, it appears that the vapor samples are anti-inflammatory. Taken together, although there is little doubt that air pollutants have deleterious effects on human health; the vapor phase appears to have a protective effect through the induction of protective proteins.

The two vapor phase samples differed in the concentration of both prooxidants and electrophiles (Table II) and in the experiments conducted the ratios of activities in the exposure media were 1.5 to 1.75 in favor of VP1. Comparison of the biological responses with the differences in

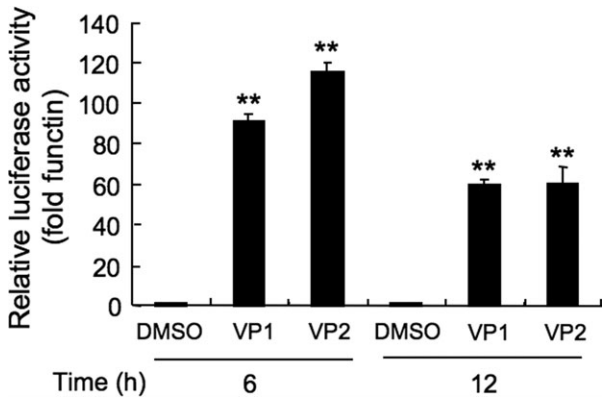

Fig. 2. Increase of ARE-dependent transcriptional activity by vapor phase samples in BEAS-2B cells. ARE-luciferase and pRL-TK cDNA-transfected cells were exposed to VP1 (2.68 m^3/mL) or VP2 (3.19 m^3/mL) for 6 or 12 h, and luciferase activity was measured by liminometer. Each value represents the mean ± SE of three independent experiments. *$p < 0.05$ and **$p < 0.01$ compared with DMSO.

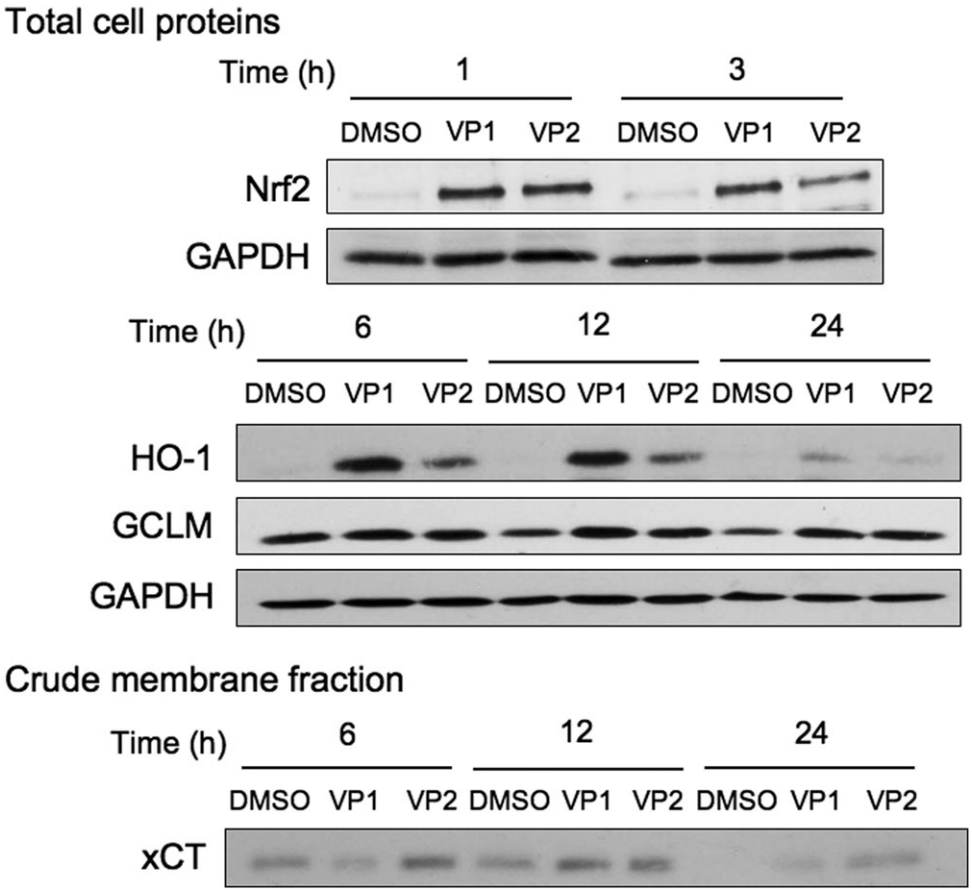

Fig. 3. Activation of Nrf2 and up-regulation of HO-1, GCLM, xCT proteins by vapor phase 3. samples in BEAS-2B cells. Cells were exposed to VP1 (2.68 m^3/mL) or VP2 (3.19 m^3/mL) for 1, 3, 6, 12, or 24 h, and total cell lysates (except xCT) or crude membrane fractions (xCT) were subjected to Western blotting with the indicated antibodies.

prooxidant and electrophile concentrations indicates responses for specific proteins are complex and the relationship to activity cannot be assessed without concentration vs. activity data. Thus, while the increase in HO-1 and xCT mRNA, the levels of activated Nrf2 and HO-1 were greater after VP1 exposure, the levels of GCLM mRNA and of Nrf2 luciferase activity as well as xCT expression were not. These apparent discrepancies are likely due to the concentration- response relationships of the individual mRNAs and proteins as well as their temporal properties, which were not investigated here.

In general, Nrf2 activity is negatively regulated by Keap1, which binds to Nrf2 and facilitates the degradation of Nrf2 via ubiquitin-proteasome system (Itoh et al., 1999). Keap1 also serves as a sensor protein and oxidative or electrophilic modification of its reactive thiol triggers Nrf2 activation (Taguchi et al., 2011). Keap1 has multiple thiol functions and we showed that vapor phase components other air samples form covalent bonds with at least one, thereby activating Nrf2 (Iwamoto et al., 2010). Consistent with that observation, treatment of these vapor samples with GSH as an alternate nucleophile partially repressed activation of the Nrf2-HO-1 pathway. A possible explanation for retention of some activity is that Keap1-independent activation of Nrf2 had occurred, for these vapor samples did induce the phosphorylation of ERK and phosphatidylinositol 3-kinase (PI3K) in BEAS-2B cells (data not shown). Direct phosphorylation of Nrf2 is also a potential mechanism of Nrf2 activation as it is a target for protein kinases such as mitogen-activated protein kinases (MAPK) and PI3K. We have reported earlier that vapor samples activate Nrf2 via electrophilic modification of Keap1 but not by production of ROS (Iwamoto et al., 2010).

In summary, the results of this study show that at concentrations of 2 to 3 m^3/mL, volatile organic species trapped by the XAD resins in ambient air samples collected in midtown Los Angeles elicit protective responses through the activation of the Nrf2-ARE pathway. The blockage of these effects by GSH and results from a prior study of vapors suggest that the responsible chemical species are likely to be electrophiles which are the dominant species in the vapor phase (Eiguren-Fernandez et al., 2010). These species also

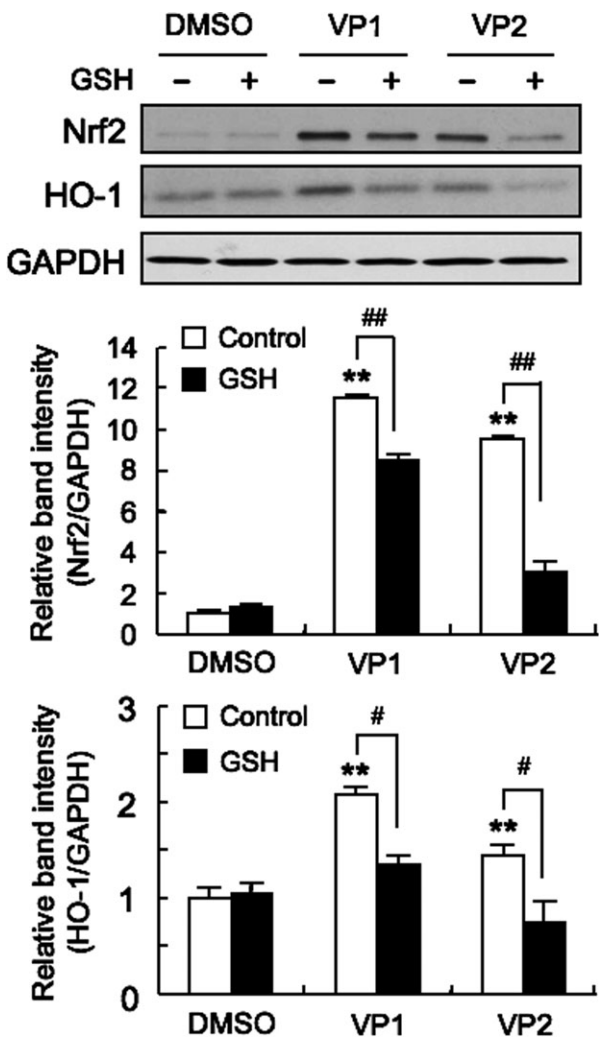

Fig. 4. Involvement of electrophiles in Nrf2 activation by vapor phase samples in BEAS-2B cells. Vapor phase samples were incubated with GSH (10 mM) for 1 h at room temperature. Cells were exposed to GSH-treated VP1 (2.68 m^3/mL) or VP2 (3.19 m^3/mL) for 3 h, and total cell lysates were subjected to Western blotting with the indicated antibodies. Each value represents the mean ± SE of three independent experiments. $^{**}p < 0.01$ compared with DMSO. $\#p < 0.05$ and $\#\#p < 0.01$ compared with control.

appear to suppress proinflammatory gene expression, particularly tumor necrosis factors which are typically increased upon exposure to ambient air and to PM fractions (Salonen et al., 2004; Delfino et al., 2010; Halatek et al., 2011). Thus, the vapor phase components of ambient air contain chemical species that affect the overall response to exposure and should be monitored as well as the particle phase.

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