Journal of Toxicology and Environmental Health, Part A, 70: 1731–1744, 2007
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ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287390701457746

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Effects of $PM_{2.5}$ Collected from Cache Valley Utah on Genes Associated with the Inflammatory Response in Human Lung Cells

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In January 2004, the normally picturesque Cache Valley in northern Utah made national headlines with the highest $PM_{2.5}$ levels in the nation. Epidemiological studies linked exposure to particulate air pollution in other locations with stroke and Alzheimer's disease and to early mortality from all causes, cancer, and cardiopulmonary diseases. To determine potential effects of these particles on human health, human bronchial epithelial cells (BEAS-2B) were cultured with $PM_{2.5}$ collected from various locations in the Cache Valley. These particles were slightly cytotoxic, but more potent than NH_4NO_3, the major chemical component of Cache Valley $PM_{2.5}$. Gene expression analysis of $PM_{2.5}$-exposed cells was performed using microarray and quantitative reverse-transcription polymerase chain reaction (RT-PCR.) Among other genes, $PM_{2.5}$ exposure induced genes and proteins involved in the inflammatory response. Most notably, $PM_{2.5}$-exposed cells showed significant gene level upregulation of activating receptors to interleukins 1 and 6 (IL-1R1 and IL-6R), as well as concomitant increases in protein. Increases in IL-1 receptor associated kinase-1 (IRAK) protein were observed. $PM_{2.5}$ exposure resulted in release of IL-6, as well phosphorylated STAT3 protein, providing evidence that PM activates the IL-6/gp130/STAT3 signaling pathway in BEAS-2B cells. IL-20 and major histocompatibility complex peptide class-1 (MICA) were upregulated and cleavage of caspase-12 was detected. In total, our results indicate that Cache Valley $PM_{2.5}$ produces the upregulation of important cytokine receptors and is able to activate both IL-1R- and IL-6R-mediated signaling pathways in human lung cells. These observations are generally consistent with the adverse effects associated with inhalation of fine particulate matter like $PM_{2.5}$.

Received 10 November 2006; accepted 8 March 2007.

The authors thank Lorinda Anderson and Dr. John Veranth of the University of Utah for performing the IL-6 ELISA and Dr. Jeffrey Hall and John Guarisco for their critical reviews of the article. This work was supported in part by grants from the Marriner S. Eccles Foundation, the Utah Water Research laboratory, the Center for Integrated Biosystems at Utah State University, and by the Utah Agricultural Experiment Station, where it is paper number 7830.

Portions of this report were presented at the 45th annual meeting of the Society of Toxicology, San Diego, CA, March 2006 (Abstract 237).

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The Cache Valley in northern Utah/southeastern Idaho has an area of 3050 km^2 with a 2004 population estimate of 100,000 (USCB, 2006). The valley floor averages 1430 m above sea level and is surrounded by steep mountain ranges with elevations as high as 3042 m (Malek et al., 2006). During winter months, this narrow valley is susceptible to shallow temperature inversions where high barometric pressure traps cold air within the valley basin. Such meteorological phenomena lead to the accumulation of pollutants, resulting in episodic increases in particulate (PM) concentrations, such as the reading of 132.5 $\mu g/m^3$ $PM_{2.5}$ that occurred in January 2004 and was described as "worst in the nation" (Malek et al., 2006).

While only partially chemically characterized, secondary nitrate salts, specifically NH_4NO_3, are important components of Cache Valley $PM_{2.5}$ (Martin & Koford, 2005) and are formed from the primary pollutants NOx (principally from automobile exhaust) and ammonia from livestock excreta. This reaction is catalyzed by cold temperatures, high humidity, volatile organic compounds (VOC), and the presence of other reactive compounds. Nitrate salts may be coupled to an organic component (<25%), which may enhance condensation onto the particle. Cache Valley $PM_{2.5}$ also contains sulfites and crustal components, with the latter accounting <6%; of particular toxicological significance is that the majority (>80%) of these fine particles have a mean geometric diameter <1 μm (Mangelson et al., 1997; Silva, 2004; Martin & Zhu, 2004; Silva et al., 2004). The mass concentration and chemical makeup of Cache Valley $PM_{2.5}$ have been shown to be spatially and temporally consistent during the

winter (Martin & Zhu, 2004; Martin & Koford, 2005). Unlike other Utah urban areas where some fine particles are crustal elements drifting from nearby deserts (Pope et al., 1999), Cache Valley particles are generated locally.

Epidemiologic studies have associated exposure to fine particulates with early mortality (Schwartz et al., 1996, 2002; Klemm et al., 2000) by stroke (Hong et al., 2002), cancer (Dominici et al., 2005), cardiopulmonary (Pope et al., 2002; Krewski et al., 2005) and cardiovascular disease (Delfino et al., 2005; Ulrich et al., 2002). Particulate air pollution has also been associated with hospital admissions for cardiovascular and respiratory diseases (Dominici et al., 2006). Short-term exposures to air pollution are sufficient for increases in mortality (Ostro et al., 2006). While no epidemiologic studies examined the effects of particulate air pollution in Cache Valley, studies examining particulate air pollution in other Utah urban centers have associated exposure with early mortality (Pope et al., 1999) from a variety of cardiovascular and other diseases (Pope & Dockery, 2006).

In humans, the bronchial epithelium acts as a physicochemical barrier and plays a crucial role in initiating and augmenting defense mechanisms and is responsible for signaling a systemic response (Mills et al., 1999). Of particular relevance to this investigation is that airway epithelial cells exposed to PM in vivo and in vitro are known to initiate and amplify the inflammatory response by secreting a number of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 (Dick et al., 2003; Veranth et al., 2006). It was therefore hypothesized that Cache Valley $PM_{2.5}$ has the potential to elicit inflammatory or cytotoxic responses in human bronchial epithelial cells. In this study, the effects of Cache Valley $PM_{2.5}$ were examined on some inflammatory and cytotoxic mechanisms in cultured human bronchial epithelial (BEAS-2B) cells.

METHODS

Chemicals and Reagents

BEAS-2B cells were a gift from Dr. Katherine Macé (Nestle Research Centre, Lausanne, Switzerland). BEAS-2B cells are classified as biosafety level 2 (BSL-2) and appropriate precautions were observed. Trypsin/ethylenediamine tetraacetic acid (EDTA), HEPES buffered saline solution (HBSS), and trypsin neutralizing solution (TNS) were from Cambrex (Walkersville, MD). LHC-9 cell growth media came from Invitrogen (Camarillo, CA). FastStart TaqMan Probe Master mix and hydrolysis probes were from Roche (Indianapolis, IN). Interleukin 1 receptor type 1 (IL-1R1) and interleukin 6 receptor (IL-6R) primary antibodies were purchased from R&D Systems (Minneapolis, MN). Anti-IRAK-1 primary antibody was from Affinity Bioreagents (Golden, CO). Biotinlyated protein ladder, anti-rabbit and anti-biotin secondary antibodies, IFN-γ treated HeLa cell extract, caspase-12, caspase-3, TNF-α, and Phospho-STAT3 primary antibodies were obtained from Cell Signaling (Beverly, MA). CaspACE Colorimetric caspase-3/7 activity detection kit was from Promega (Madison, WI). Millipore Ultrafree DA spin columns (Millipore, Billerica, MA) and Corning well cell culture cluster 96-well plates were obtained from Fisher Scientific (Pittsburgh, PA). Quick Start Bradford protein assay was from Bio-Rad (Hercules, CA). The *Limulus* amebocyte lysate (LAL) assay QCL-1000 was from Cambrex (East Rutherford, NJ).

$PM_{2.5}$ Collection and Treatment

Samples of ambient $PM_{2.5}$ were collected from one urban ("Smithfield") and two rural ("Evan's Farm," and "Cornish") Cache Valley locations, following the Federal Reference Method outlined in the U.S. Code of Federal Regulations (40 CFR 50, 2006). The official particulate concentrations for the county reported to the U.S. Environmental Protection Agency (EPA) came from the Utah Department of Environmental Quality Division of Air Quality and were measured in nearby Logan, UT. The collectors used were an Airmetrics MiniVol (Eugene, OR) set to collect $PM_{2.5}$ (the collector can collect PM_{10} and $PM_{2.5}$ but not simultaneously) for the rural locations, or an RAAS-1000 $PM_{10}/PM_{2.5}$ sampler (Anderson, Atlanta, GA), which separates atmospheric particulate matter into the desired size fractions. A U.S. EPA-designed WINS impactor was used to collect $PM_{2.5}$ onto Whatman 47-mm PTFE air monitoring membrane filters (model 7592–104). The instruments were programmed to operate for 24-h periods (midnight to midnight) at flow rates of 5 L/min (lpm; rural) and 16.7 lpm (urban). Each filter was stored in individual plastic petri dishes in a room-temperature desiccator until equilibrated to within ± 2.5 μg before and after collection. Filters were handled only with Teflon-coated, stainless-steel forceps. The collectors were cleaned prior to seasonal setup, and the impactor was removed and cleaned following episodes of high PM. PM was collected from November to January 2004, January 2002, and 2003. Unless otherwise stated, the filters used were from the Smithfield location.

For extraction, filters were weighed then placed into 50-ml conical bottom Falcon tubes (Becton Dickenson, Franklin Lakes, NJ) and immersed in 30-ml autoclaved and filtered (0.1 μm) deionized H_2O. Tubes containing the filter and water were then sonicated for 90 min in a sonicator bath (model 1200 Branson Ultrasonics, Danbury, CT). Tubes and filters were dried and weighed to determine the amount of $PM_{2.5}$ extracted. In most cases the filters were visibly darkened following collection and the appearance was unaltered following extraction. Following desiccation a film was observable on the bottom of the Falcon tubes. Extracted $PM_{2.5}$ was then resuspended in 1 ml LHC-9 cell growth media and used immediately or stored at 4ºC until used (generally within 16 h). The extraction efficiency of $PM_{2.5}$ from the filters was routinely 75–80%. Filters were discarded following extraction.

Cytotoxicity, Endotoxin Detection, and Apoptosis Assays

Cytotoxicity was measured using a variation of the MTT method (Mosmann 1983) as described in (Van Vleet et al., 2002) with the exception that 36 μl of 0.1 *N* HCl in isopropanol was

added to dissolve crystals and neutralize phenol red rather than dimethyl sulfoxide (DMSO). The reactions were read on a microplate reader (Labsystems Multiskan MCC/340 Thermoelectron, Pittsburgh, PA). Staurosporine (1 μM) or caffeine (250 μM) (Van Vleet et al. 2006) were used as positive controls. Cytotoxicity of a range of $PM_{2.5}$ (5.5–1101 $\mu g/m^3$) collected on separate occasions and locations were tested, as well as that of NH_4NO_3, the principal component of wintertime Cache Valley $PM_{2.5}$. Eight replicates of each concentration were tested. Differences from control were determined using Holm–Sidak one-way analysis of variance using SigmaStat (SPSS, Chicago).

The introduction of artifactual endotoxin was examined in $PM_{2.5}$ extraction water, cell growth media, and a blank, extracted PTFE filter using the LAL assay kit. Samples of collected particulate material at two concentrations (1.2 μg/ml $PM_{2.5}$ and 5.3 μg/ml PM_{10}) were also examined.

Caspase-3 protein and activation were determined using the CaspACE colorimetric assay kit according to the manufacturer's instructions.

Cell Culture

Cells were seeded into 10 ml of LHC-9 in T-75 flasks and incubated at 37°C/5% CO_2 with media changes the following day and alternate days thereafter. Cells from the same passage were counted and aliquoted to 6.15×10^4 cells per flask, grown to approximately 70% confluence then cultured 24 h with a range of $PM_{2.5}$ concentrations (0.13–3.90 μg/ml). After exposure, media was removed, cells washed with 5 ml HBSS, detached with 5 ml 0.25mg/ml trypsin/EDTA, and pelleted in TNS.

Microarray RNA Analysis

Following cell harvest, total RNA was extracted using the RiboPure RNA isolation kit (Ambion, Austin, TX) according to the manufacturer's instructions including the optional DNAse incubation and purification step. RNA was then quantified spectrophotometrically (A_{260}) (model ND-1000 spectrophotometer Nanodrop, Wilmington, DE), then analyzed for gene expression by microarray. Total RNA from cultures exposed 24 h to $PM_{2.5}$ and control, was submitted to an on-campus core facility where it was analyzed for quality (Bioanalyzer, Agilent, Palo Alto, CA). Gene expression was examined using Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA). Array images were analyzed using Array Assist (Stratagene, La Jolla, CA) software applying the PLIER algorithm. Genes of interest were limited using exposure/control ratios of >2 (Koike et al., 2004). Genes were further limited by selecting those that exhibited a >2 ratio across all treatments using Microsoft Excel (Redmond, WA) and were linked to known proteins using NetAffx software (Affymetrix, Santa Clara, CA). While a variety of genes met these criteria, genes related to inflammation, stress response, and cell death were selected. Verification of the array data was performed using quantitative reverse-transcription polymerase chain reaction (qRT-PCR).

qRT-PCR Analysis

Total RNA from $PM_{2.5}$-exposed and control cells was isolated as described earlier. One microgram total RNA was used to transcribe cDNA using the 2.5 μ*M* anchored-oligo$(dT)_{18}$ primer with the Transcriptor First Strand cDNA synthesis kit (Roche, Indianapolis, IN). A control cDNA synthesis reaction lacking reverse transcriptase was run for each reaction. cDNA was then stored at −20°C until used for qRT-PCR. High-performance liquid chromatography (HPLC)-purified intron spanning primers were designed using the Universal ProbeLibrary assay design center (Roche, Indianapolis, IN) and ordered from Integrated DNA Technologies (Coralville, IA). Primers and probes are given in Table 1. All PCR reactions were run using the following cycling parameters: an initial melting and *Taq* antibody deactivation step at 95°C for 10 min

TABLE 1
RT-PCR Primers

Name	Locus ID	Forward primer	Reverse primer	Primer concentration (n*M*)	Probe	ProbeLibrary probe number
Homo sapiens MHC class I polypeptide-related sequence A (MICA)	NM_000247.1	ggcatcttccctt ttgcac	ggacagcacc gtgaggttat	100	ggagctg	24
Homo sapiens interleukin 20 (IL20)	NM_018724.3	acagccagattc tgagtcacttt	ctagttcccccca aagccttc	200	caggcagc	27
Homo sapiens interleukin 6 receptor (IL6R), transcript variant 1	NM_000565.2	cacattcctggtt gctgga	cagcttccacgt cttcttga	600	ctcctctg	82
Homo sapiens interleukin 1 receptor, type I (IL1R1)	NM_000877.2	tgttcatttatgga agggatga	ttctgcttttcttta cgttttcatt	400	tggggaag	60

followed by 40 cycles of a melting step at 95°C for 15 s and a 60°C annealing/extension step for 1 min. Optimal primer concentrations for all examined genes were determined by running a 50- 700-n*M* equimolar primer concentration range with a constant 1 µl cDNA in a 25-µl reaction using the FastStart TaqMan Probe Master mix. Reactions were separated on 2% agarose gels (EC Minicell Primo, Fisher Scientific, Pittsburgh, PA) at 150 V for 20 min. PCR product images were analyzed and archived using a model 920 imager and Labworks software (UVP, Upland, CA). After the optimal primer concentration was selected, the PCR product was excised from the gel and purified using Millipore Ultrafree DA spin columns for sequencing and use as a standard. PCR product standard DNA was quantified spectrophotometrically (Thermospectronic Genesys 6, Fisher Scientific, Pittsburgh, PA) and copy concentration was determined using the molecular weight of the known amplicon DNA sequence as determined by OligoAnalyzer software (IDT, Coralville, IA). Standard DNA was diluted 10^3, then serially diluted in triplicate to generate a standard curve. Quantitative reactions were done in triplicate including no cDNA and no reverse transcriptase controls using a DNA Engine Opticon 2 thermal cycler (Bio-Rad, Hercules, CA) using the already mentioned reaction conditions with the FAM read step occurring after the 60°C annealing/extension step. Each reaction was performed with 110 n*M* probe concentration and optimized primer concentration. Quantification was performed based upon the standard curve and calculated cycle threshold (C_T) values by the OpticonMonitor 2.02 software included with the instrument. Differences in mean values for treated vs. control were determined using Holm–Sidak one-way analysis of variance with SigmaStat software (SPSS, Chicago).

Protein Detection Assays

Western blotting was performed as previously described (Van Vleet et al., 2006). In brief, media from cell culture was removed, centrifuged, and frozen for subsequent analysis. Cells were washed with 5 ml HEPES buffered saline solution and harvested via trypsinization. Cells were resuspended in 100 µl CHAPS cell extract buffer and total protein was quanitified. Equal protein concentrations were loaded onto gels, electrophoresed, transferred to nitrocellulose membranes, probed with the appropriate primary and secondary antibodies, and visualized using SuperSignal West Femto chemiluminescent substrate (Pierce, Franklin Lakes, NJ) using a Nucleotech Imaging workstation (Hayward, CA). Densitometric analysis was performed using the Histogram function in PhotoShop CS (Adobe, San Jose, CA) (Woznicova, 2001). The software provided mean pixel intensity, standard deviations, and total number of pixels, which were sufficient for one-way analysis of variance using the Holm–Sidak test using significance test at $p < .05$. TNF-α release was examined using cells treated with higher concentrations of Cache Valley $PM_{2.5}$ (≤25 µg/ml) using the R&D Quantikine enzyme-linked immunosorbent assay (ELISA) kit (Minneapolis, MN) according to the manufacturer's instructions with 6 replicates per treatment. Measurement of IL-6 release into the media was performed as described (Veranth et al., 2006).

RESULTS

PM Extraction, Cytotoxicity, and Endotoxin Detection

Extraction of PM ranged from 70 to 670 µg from more than 10 filters. A prior ethanol wash resulted in postextraction filter weights that were less than the precollection filter weights; hence extractions were done without an ethanol wash. Most of the extracted PM was used for cytotoxicity. For subsequent gene expression experiments, cells were exposed to $PM_{2.5}$ at concentrations below those found to be cytotoxic. A single filter generally yielded sufficient PM for two independent experiments. Due to the influence of weather patterns on PM conditions (Malek et al., 2006), samples taken on different days were not pooled.

The endotoxin concentrations in LHC-9 media, extraction water, and an extract of a blank collection filter were below the detection threshold (<0.1 EU/ml) of the LAL assay. A 1.2-µg/ml sample of $PM_{2.5}$ also contained <0.1 EU/ml endotoxin. Endotoxin above 0.1 EU/ml (0.15 EU/ml) was detected in a 5.3-µg/ml sample of PM_{10} collected on the same day as the $PM_{2.5}$ (not used in this study).

Our data indicate that exposure to Cache Valley $PM_{2.5}$ resulted in minimal observable cytotoxicity in BEAS-2B cells, as determined by MTT. Figure 1A shows the concentration dependency of cytotoxicity of $PM_{2.5}$ collected from an urban sampler in the city of Smithfield, UT. Cells were cultured with varying concentrations of PM collected on January 14, 2004, when the official ambient concentration was 116.9 µg/m^3 and with the highest concentration (1101.4 µg/ml) producing 39% inhibition. No significant differences in cytotoxicity were observed from $PM_{2.5}$ obtained from two different rural samplers on the same day and exposed to the same concentrations (data not shown). Even though Cache Valley $PM_{2.5}$ was only modestly cytotoxic, it was substantially more potent than NH_4NO_3, when compared on a mass per volume basis (Figure 1B). NH_4NO_3 resulted in MTT conversion significantly above control at most concentrations, with inhibition occurring only at the highest concentrations of 10 and 100 m*M* (800.4 and 8004 µg/ml).

Gene Expression Analysis, IL-6 Release, and Protein Activation

Selected upregulated genes related to the inflammatory response determined by microarray analysis are presented Table 2. MICA, IL-20, IL-1R1, and IL-6R were selected for verification of mRNA upregulation by qRT-PCR. In many

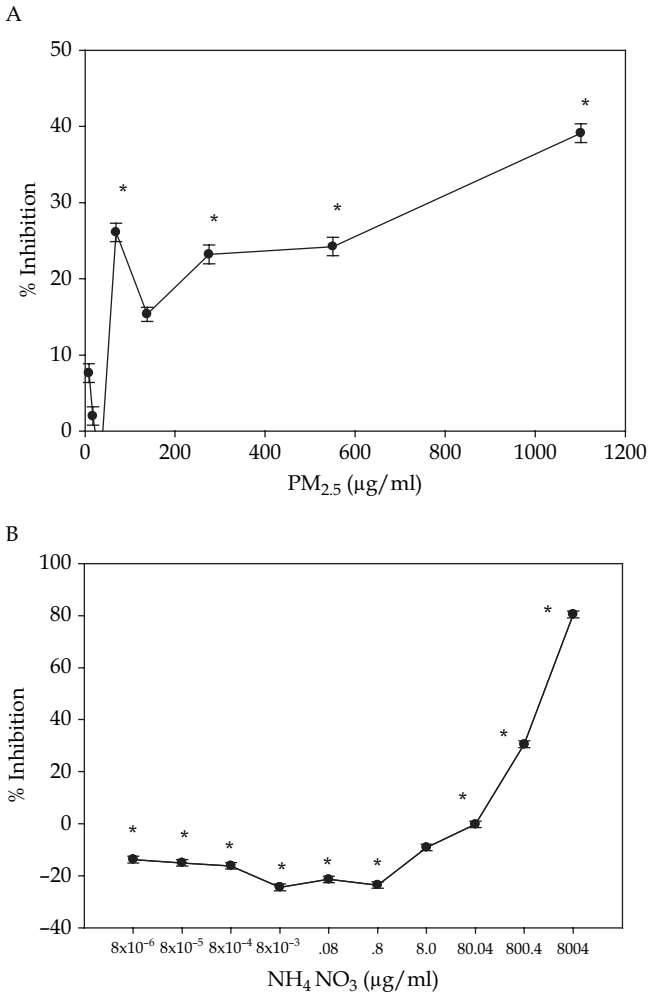

FIG. 1. Cytotoxicity resulting from 24-h exposures to Cache Valley $PM_{2.5}$ in BEAS-2B cells as determined by the MTT assay. (A) Cytotoxicity of $PM_{2.5}$ samples collected in Smithfield, UT, January 14, 2004, when the official State ambient $PM_{2.5}$ reading for the County was 116.9 μg/m^3. (B) By comparison, NH_4NO_3, a principal component of Cache Valley $PM_{2.5}$, was substantially less cytotoxic than $PM_{2.5}$ in that it stimulated MTT metabolism at concentrations <8 μg/ml (100 μ*M*) with inhibition only occurring at the highest concentrations >80.04 μg/ml (1000 μ*M*). Multiple (n = 16) assays were run, of which these are representative. Each point is mean (n = 8) ± SD. Asterisk indicates significant difference from control as determined by Holm–Sidak one-way analysis of variance on ranks ($p < .05$).

cases, $PM_{2.5}$ exposure produced a significant upregulation of these genes. For example, there was a significant upregulation of mRNA coding for IL-1R1 in cells exposed to all but the lowest $PM_{2.5}$ concentration (0.69 μg/ml) (Figure 2). Conversely, there also was significant upregulation of IL-20 mRNA, but only in cells exposed to the lowest $PM_{2.5}$ concentration, while MICA was significantly upregulated in a concentration-dependent manner in cells treated with 1.37, 2.72, or 4.03 μg/ml $PM_{2.5}$ (Figure 2). The lack of concentration dependency of the IL-20 response was repeatedly verified using 3 different reverse-transcriptase (RT) reactions.

There was a significant upregulation of IL-6R in cells cultured at all $PM_{2.5}$ concentrations (Figure 2). These results were verified by reactions involving a minimum of two RT steps. To confirm that increases in expression were not due to differential reverse transcriptase activity, the content of single-stranded (ss) DNA after the cDNA synthesis step was determined. The control cDNA reaction contained more ssDNA than any of the treatments (1.1–1.9 × more in both RT reactions); thus, any increases in mRNA expression could not be attributed to increased cDNA from differential RT activity. Expression of IL-6R and IL-1R1 proteins was evaluated by Western blotting to confirm that the increases in gene expression resulted in increased protein levels. There was an increase in IL-6R and IL-1R1 protein levels, which occurred at all $PM_{2.5}$ concentrations (Figures 3 and 4). Because IL-1R1 is essential for IL-1 signaling (Sims et al., 1993) and involves interleukin 1 receptor associated kinase 1 (IRAK), the effect of $PM_{2.5}$ was examined on expression of IRAK protein. Microarray detected IRAK upregulation in the unmodified (CHP) data set applying the same selection (>2) criteria, but not when data were transformed with the PLIER algorithm (data not shown). Western blots showed a significant upregulation of IRAK protein levels in cells exposed to all concentrations of $PM_{2.5}$, and the expression pattern with respect to PM concentration appeared similar to that of IL-1R1 (Figure 4).

Cells treated with $PM_{2.5}$ concentrations of 0.65, 1.3, or 3.9 μg/ml released significantly higher levels of IL-6 into the media (Figure 5). There was no detectable TNF-α release into the cell media after 24 h of $PM_{2.5}$ exposure. Subsequent Western blotting revealed the presence of the full-length 32-kD TNF-α precursor but not the 17-kD mature TNF-α (data not shown). Because of the effect of PM on activation of the IL-6 signaling pathway, studies were performed to examine the expression of phosphorylated STAT3 protein in PM-exposed cells using a primary antibody specific for tyrosine 705-phosphorylated STAT3. Figure 3 shows that there was a clear concentration-dependent increase in levels of phosphorylated STAT3 in cells exposed to Cache Valley $PM_{2.5}$. The antibody detects doublet bands of 79 and 86 kD.

Given the observation of PM-related upregulation of genes associated with apoptosis by microarray, the presence and activation of both caspase 12 and caspase 3 in control and PM-exposed cells were determined. Figure 6 shows that $PM_{2.5}$ exposure produced increased expression of the 60-kD procaspase-12 protein as well as activation of caspase-12, as evidenced by the presence of the 25-kD caspase-12 cleavage product compared to control cells. In contrast, while caspase-3 protein was significantly higher in $PM_{2.5}$-treated cells, there was no detectable activation of caspase-3 as shown by the lack of the 17-kD cleavage product using Western blotting or by the use of an alternate colorimetric activity assay (Figure 7).

TABLE 2
Selected Upregulated Genes From Array Analysis

Protein	Function	Ratio dose/control		
		0.13 μg/ml	2.65 μg/ml	3.90 μg/ml
Major histocompatibility complex class I polypeptide (MICA)	Response to stress Cellular defense response Cell recognition Antigen presentation	6.56	4.48	5.09
Interleukin 20 (IL-20)	Immune response Hemopoiesis Positive regulation of phosphorylation of Stat3 protein Positive regulation of epidermal cell differentiation Regulation of inflammatory response Positive regulation of keratinocyte differentiation	17.93	2.04	7.29
Interleukin 1 receptor, type I (IL-1R1)	Inflammatory response Cell surface receptor linked signal transduction	5.42	2.57	2.57
Interleukin 6 receptor (IL-6R)	Immune response Cell proliferation Development Cell surface receptor linked signal transduction	2.84	2.19	2.48
Toll-like receptor 5 (TLR-5)	Inflammatory response	22.59	2.41	16.52
Caspase 3, apoptosis-related cysteine protease	Proteolysis and peptidolysis Apoptosis and induction of apoptosis	17.48	2.71	9.81
Toll interacting protein (TollIP)	Inflammatory response Intracellular signaling cascade Phosphorylation Immune cell activation	10.21	2.31	12.56

DISCUSSION

Laboratory research determining the potential adverse human health effects of fine particulate air pollution has been stimulated by an increasing number of epidemiologic studies in several geographic locations that have consistently associated exposure to this class of air pollutants with a variety of cardiovascular diseases (Krewski et al., 2005). Exposure to airborne particulates in nearby Utah and Salt Lake Valleys (80 and 120 miles south of Cache Valley, respectively) during similar meteorological episodes was associated with increases in early mortality and hospital admissions due to cardiovascular diseases (Pope & Dockery, 2006). That many of the adverse effects associated with fine particle exposure are outside the respiratory tract may be due to signaling and signal amplification from lung cells, or to the ability of small particles to penetrate the central circulation or the brain (Nemmar et al., 2002; 2004; Oberdorster et al., 2004).

Our results demonstrate that while Cache Valley $PM_{2.5}$ was only modestly cytotoxic in cultured human BEAS-2B cells, it induced upregulation of several genes associated with the inflammatory response as well as increased STAT3 activation. The cytotoxicity of $PM_{2.5}$ was substantially greater than that of NH_4NO_3, a principal chemical component. In fact, low ($\leqq 1$ μ*M*) NH_4NO_3 concentrations exhibited a significant stimulatory effect on MTT reduction, which may be explained by increases in cytoplasmic conversion of MTT (Vistica et al., 1991) or in mitochondrial respiration (Gerlier and Thomasset, 1986). Increased MTT reduction was shown to occur without an increase in cellular growth (Gerlier and Thomasset, 1986). Minimal cytotoxicity of fine particulates in vitro was also observed in various cell types by other researchers (Monn and Becker, 1999; Pozzi et al., 2003). While NH_4NO_3 may play a synergistic role in some of the other observed effects, it seems reasonable that the cytotoxic properties of Cache Valley $PM_{2.5}$ are not primarily due to this compound.

More significantly, the data showed that the cellular response to exposure to our locally-collected $PM_{2.5}$ appears to involve both IL-1R- and IL-6R-mediated pathways. Interleukin-1 and IL-1R1 play essential roles in illnesses associated with PM exposure such as stroke, progressive neurodegeneration, mild asthma, chronic obstructive pulmonary disease

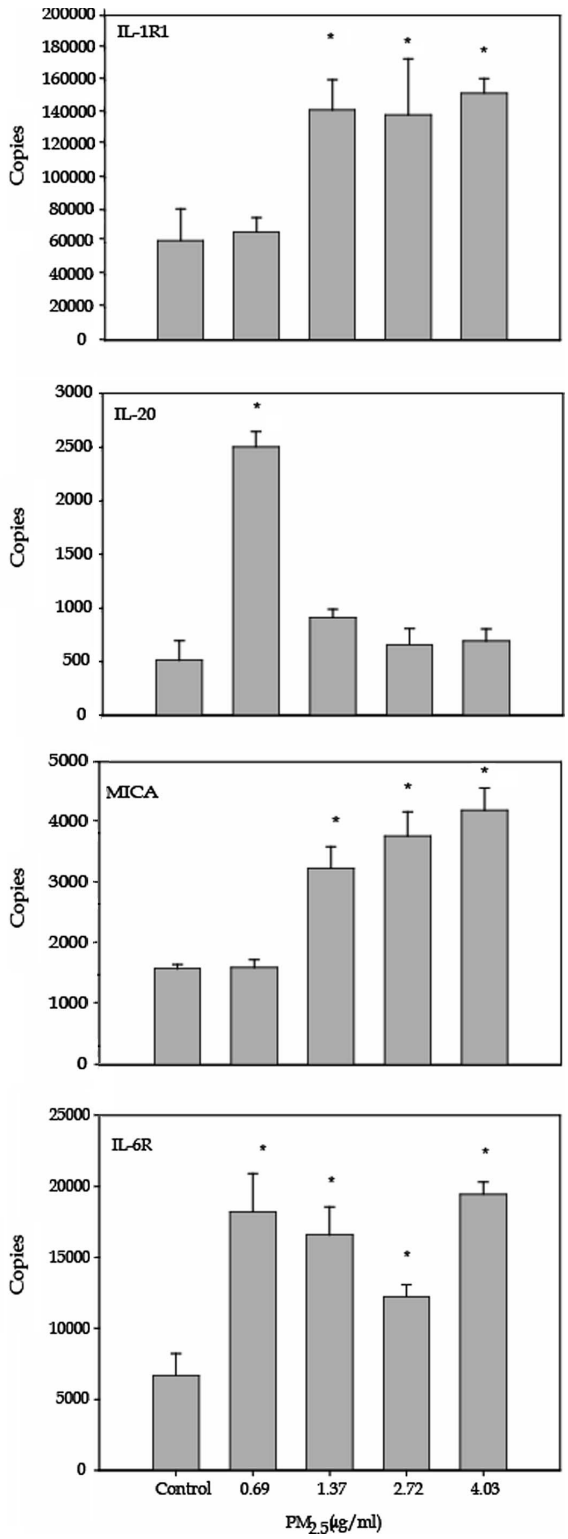

FIG. 2. Absolute RT-PCR quantification of IL-1R1, IL-20, MICA, and IL-6R mRNA from BEAS-2B cells following a 24-h exposure to varying concentrations of Cache Valley $PM_{2.5}$. At a minimum, three experiments were run with each concentration in triplicate. Each bar is mean (n = 3) ± SD. Asterisk indicates significant difference from control as determined by Holm–Sidak one-way analysis of variance ($p < .05$).

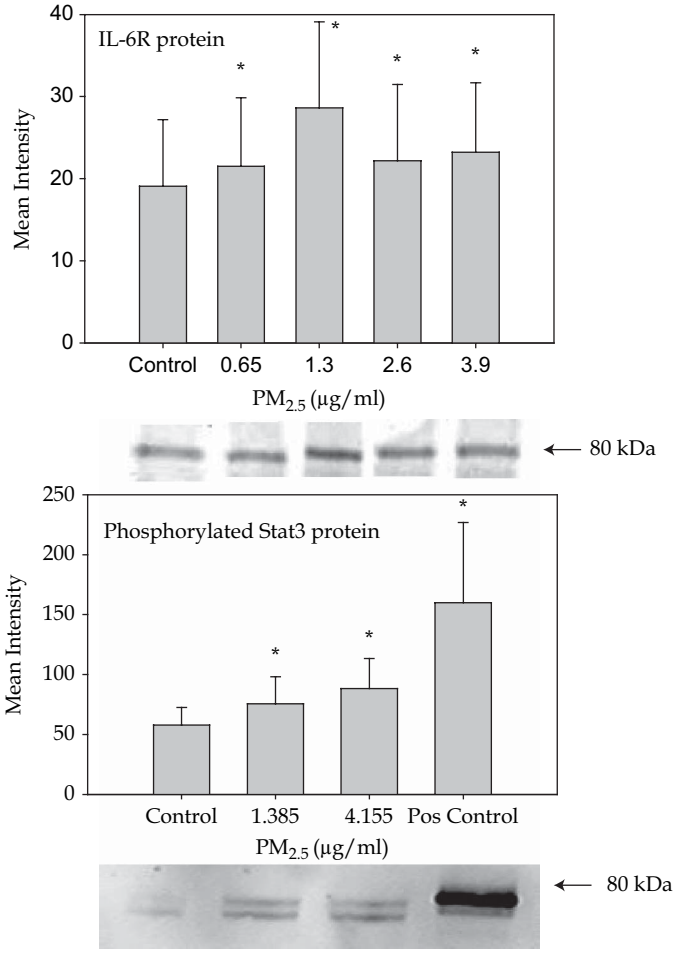

FIG. 3. Expression of IL-6R and phosphorylated STAT3 proteins in BEAS-2B cells following 24-h exposures to various concentrations of Cache Valley $PM_{2.5}$ as determined by densitometric analysis of Western immunoblots of cell extracts as described in Materials and Methods. Representative of n = 3 experiments. The IL-6 blot was loaded with 10 µg protein/lane protein, while the STAT3 blot was loaded with 4.5 µg protein/lane. There was a significant increase in cellular expression of both proteins at all $PM_{2.5}$ concentrations. The phosphorylated Stat3 antibody only detects the protein when phosphorlyated at Tyr705. Stat3 positive control is an extract of interferon-γ treated HeLa cells. In all cases, the double bands observed were consistent with the blots presented by the manufacturer. Each bar is mean band intensity ± SD. Asterisk indicates significant difference from control as determined by Holm–Sidak one-way analysis of variance ($p < .05$).

(COPD), and coronary artery disease (Basu et al., 2002, 2005; Chung, 2001; Kornman, 2006). IL-1R1 and IL-1 are involved in Alzheimer's disease (Basu et al., 2002; Griffin, 2006) and play a crucial role in atherosclerosis (Chi et al., 2004) and cardiovascular disease (Lobbes et al., 2006). Particulate air pollution stimulates IL-1 release in human monocytes (Brown et al., 2004), in alveolar macrophages (van Eeden et al., 2001), and in patients with acute lung injury (Geiser et al., 2000). The potential for IL-1R activation in PM exposed cells was supported by increased expression of its downstream signaling molecule

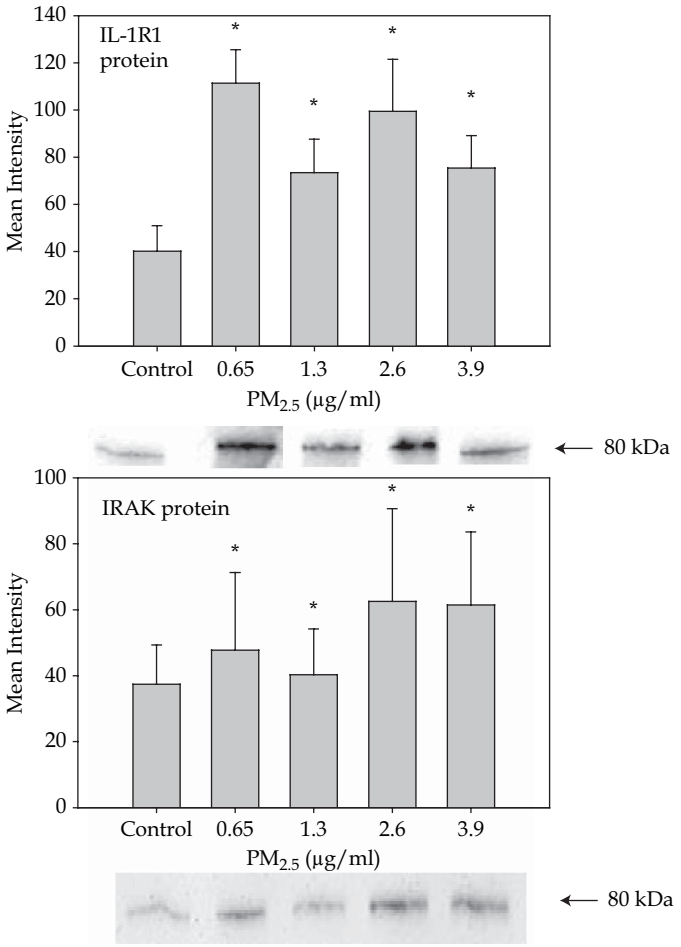

FIG. 4. Expression of IL-1R1 and IRAK proteins in BEAS-2B cells following 24-h exposures to various concentrations of Cache Valley $PM_{2.5}$ as determined by Western immunoblots of cell extracts as described in Materials and Methods. Each lane in the IRAK and IL-1R1 immunoblots was loaded with 7 µg and 10 µg protein/well, respectively. Expression of both proteins in cells exposed to all concentrations of $PM_{2.5}$ was significantly greater than in the control. Multiple (n = 3) experiments were run, of which this is representative. Each bar is mean band intensity ± SD. Asterisk indicates significant difference from control as determined by Holm–Sidak one-way analysis of variance ($p < .05$).

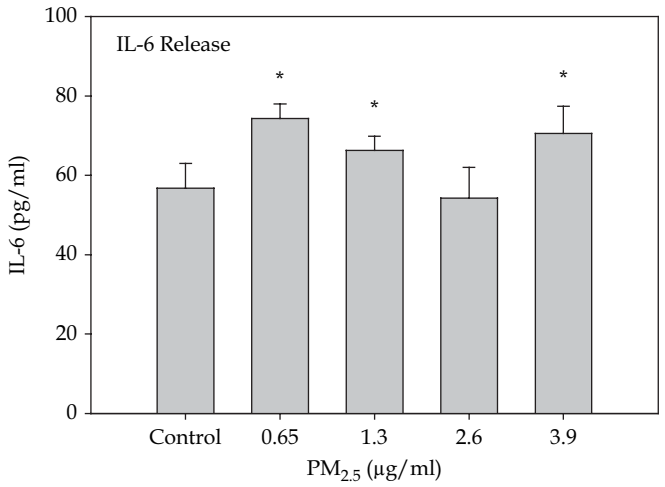

FIG. 5. Release of IL-6 from BEAS-2B cells following 24-hrexposures to various concentrations of Cache Valley $PM_{2.5}$ as determined by ELISA assay as described in Materials and Methods. Each bar is the mean IL-6 concentration (n = 9) ± SD. Asterisk indicates significant difference from control as determined by Holm–Sidak one-way analysis of variance ($p < .05$).

IRAK. Increased IRAK expression would be expected to result in heightened IRAK-mediated kinase activity, known to affect a large number of downstream cascades and transcription factors (Janssens & Beyaert, 2003; Bol et al., 2005; Takatsuna et al. 2003). Others also showed that PM exposure triggers upregulation of inflammatory receptors (TLR 2 and 4) associated with IL-1R that involve IRAK (Becker et al., 2005). Such findings are relevant in that the mechanisms for cytokine inhibition and activation are distinct. Cytokines require interaction with cell receptors to exert their effects. The effects of IL-1 are exerted exclusively through IL-R1, while inhibition of IL-1 occurs partially through binding with IL-1R2 (Rauschmayr et al., 1997). Cells responding to PM treatment by upregulating activating receptors rather than inhibitory receptors indicate that PM-mediated IL release may mediate inflammatory effects.

Likewise, increases in IL-6 and its activating receptor also are involved in PM-associated diseases. The IL-6 cytokine family is involved in a variety of cellular functions, which in addition to inflammation, include immunoglobin production, cell growth, and cell differentiation (Hirano et al., 2000). Interleukin-6R activation is directly involved in heart failure (Rivera et al., 2004), asthma, and pulmonary fibrosis (Jones et al., 2001). IL-6R also exists as a soluble receptor (sIL-6R) that enables IL-6 to potentiate effects in cells that would not normally respond to IL-6. Both differential splicing and proteolytic cleavage contribute to the production of sIL-6R, although the cause is poorly understood (Heinrich et al., 1998, 2003; Jones et al., 2001; Muller-Newen et al., 1998). Particulate air pollution was found to produce vasoconstriction and activation of the ERK kinases (Li et al., 2005), which are regulated through IL-6-gp130-STAT3 (Kamimura et al., 2003) as well as the epidermal growth factor receptor (Blanchet et al., 2004). That $PM_{2.5}$ produced IL-6 release in BEAS-2B cells was also recently observed in this cell type (Veranth et al., 2006), as well as in human monocytes (Monn & Becker, 1999) and human alveolar macrophages (Suwa et al., 2002).

Interleukin-6 signaling activity, mediated only through binding with IL-6R (via gp130 receptor) (Heinrich et al., 1998, 2003; Jones et al., 2001), leads to activation of STAT3 by phosphorylation. Our observation of STAT3 activation indicates that $PM_{2.5}$ treatment not only leads to IL-6 release, but also activates the IL-6 signaling pathway. To our knowledge, this is the first report to link particulate exposure with STAT3 activation and

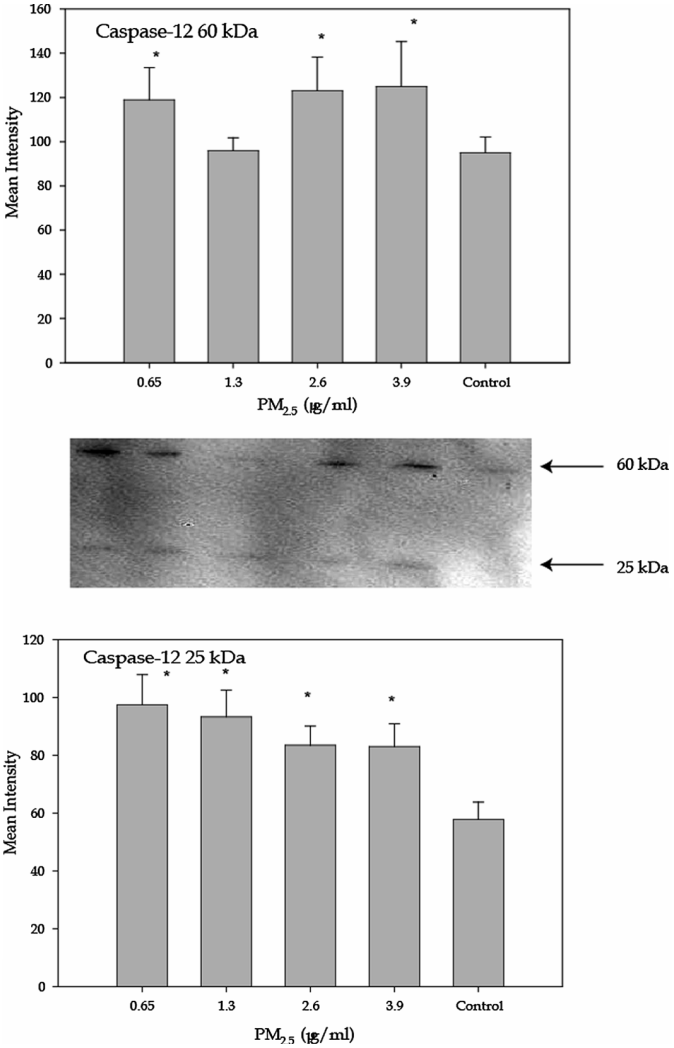

FIG. 6. Expression of caspase-12 and cleavage of caspase-12 to the 25-kD product induced in BEAS-2B cells following 24-h exposures to Cache Valley $PM_{2.5}$ as Western immunoblots of cell extracts as described in Materials and Methods. Each lane was loaded with 10 µg protein. All concentrations of Cache Valley $PM_{2.5}$ (0.65, 1.3, 2.6, and 3.9 µg/ml) induced cleavage of caspase-12. All treatments of Cache Valley $PM_{2.5}$ also yielded a significant increase in the 60-kD procaspase-12 with the exception of 1.3 µg/ml. Densitometry of the 25-kD fragment includes the control lane which had an undetectable fragment for comparison. Multiple (n = 4) experiments were conducted, of which this is representative. Each bar is mean band intensity ± SD. Asterisk indicates significant difference from control as determined by Holm-Sidak one way analysis of variance ($p < .05$).

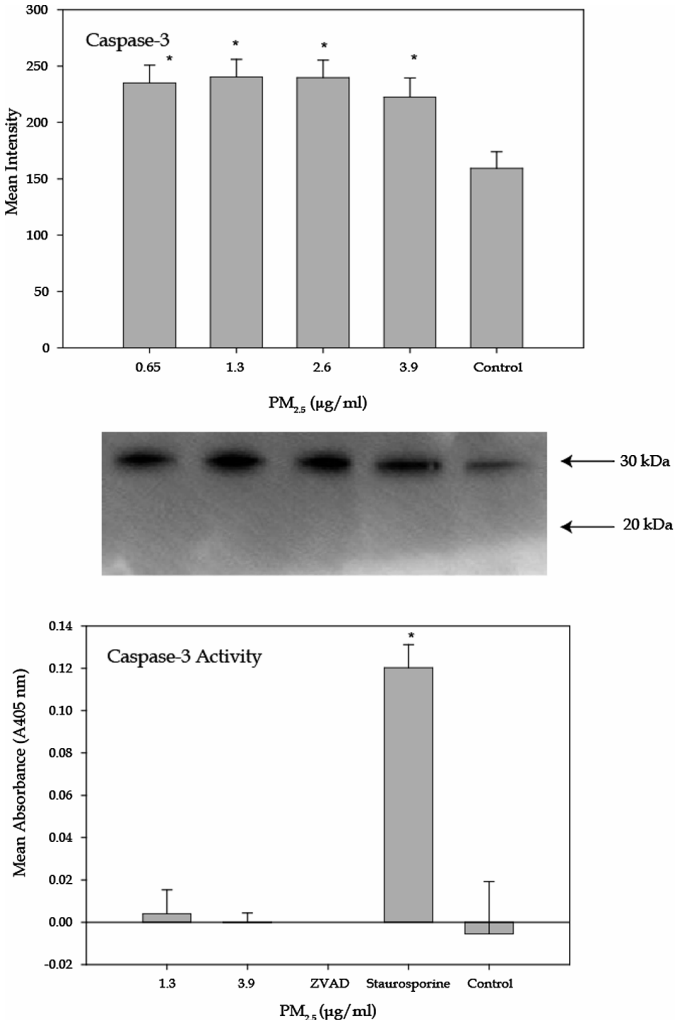

FIG. 7. Caspase-3 expression and cleavage determined by western immunoblots (top) and caspase-3 activity determined by a colorimetric assay (bottom) in BEAS-2B cells following 24-h exposures to Cache Valley $PM_{2.5}$ as described in Materials and Methods. Gels were loaded with 5 µg protein/well. While all concentrations of PM induced expression of caspase-3 protein, there was no observable 17-kD cleavage product indicative of activation of caspase-3 (top). Images were exposed to saturation to demonstrate the lack of cleavage. In the same way, there was no caspase-3 activation as determined by colorimetric activity assay (bottom). Controls include cells treated with the caspase inhibitor Z-VAD-FMK (ZVAD), 0.25 µ*M* staurosporine, and untreated cells. Results presented are the means of two different experiments with each treatment group done in triplicate. Each bar is mean absorbance (405 nm) ± SD. Asterisk indicates significant difference from control as determined by Holm–Sidak one-way analysis of variance ($p < .05$).

potential IL-6 autocrine activity in vitro. IL-20, which was observed being upregulated, may also activate STAT3 as shown in human keratinocytes (Blumberg et al., 2001). IL-20 gene expression was only induced significantly at the lowest PM concentration (0.69 µg/ml) (Figure 2), while phosphorylated STAT3 levels were altered in a $PM_{2.5}$ concentration-dependent manner (Figure 4). An explanation as to why IL-20 transcription only occurred at the lowest concentrations over multiple experiments is not readily available. The copy number was low and these cells may not release it. While the promiscuity of the gp130 receptor (Dahmen et al., 1998; Jones et al., 2001) precludes the assumption that the observed STAT3 activation was solely due to IL-6 autocrine activity (Yeh et al., 2006), the increases in STAT3 phosphorylation were undoubtedly due to $PM_{2.5}$ treatment. Phosphorylated STAT3 was detected in the

control cells. Phosphorylated STAT3 is constitutively expressed in some cell types (Kube et al., 2001), and this also may be case in BEAS-2B cells. (Veranth et al., 2006) That Cache Valley $PM_{2.5}$ activated STAT3 illustrates the complexity of IL-6 signaling. While IL-6 activation is involved in the already-mentioned adverse health affects such as heart failure, IL-6 is also anti-inflammatory (Opal & DePalo, 2000) and responsible in part for the beneficial health effects of exercise (Petersen & Pedersen, 2005). STAT3 activation is the dominant mediator in the anti-inflammatory effects of IL-10 (Williams et al., 2004), is protective against hyperoxia in the lung (Lian et al., 2005), and is necessary for cardioprotection following cardiac hypertrophy (Butler et al., 2006). However, autocrine IL-6/STAT3 activation is involved in adenocarcinoma pathogenesis (Yeh et al., 2006). STAT3 is considered to be an oncogene (Bromberg et al., 1999) involved in a number of cancers (Hodgeet al., 2005) and is a target for chemotherapy (Leeman et al., 2006). Given the association of PM with lung cancer mortality (Krewski et al., 2005), the ability of PM to increase activation of STAT3 and potentially other oncogenes is important and the subject of ongoing research. STAT3 activation has been shown to inhibit TNF-α production (Nishiki et al., 2004) and release (de Jonge et al., 2005). This may explain why this study and others (Veranth et al., 2004) were unable to detect TNF-α release in PM-treated BEAS-2B cells. Western blotting revealed the full-length inactive form of TNF-α, indicating that it is synthesized by these cells (data not shown). These cells have been shown to release TNF-α when treated with studded-tire wear particles (Lindbom et al., 2006) and following *Streptococcus pneumoniae* infection (Schmeck et al., 2006). It is possible that the inability to detect TNF-α may be due to the cytokine binding directly to the particles as occurs with diesel exhaust PM (Seagrave et al., 2004). However, the ELISA kit used is capable of detecting TNF-α bound to soluble receptors, as well as free TNF-α.

In addition to IL-20, Cache Valley $PM_{2.5}$ induced the expression of MICA, which is a recognized response to stress and pathological conditions (Borchers et al., 2006). While not directly related to activation of IL-1R or IL-6, MICA expression further indicates induction of cellular stress by $PM_{2.5}$.

Particulate air pollution triggers calcium release into the cytoplasm (Brown et al., 2004), which ultimately activates caspase-12 (Nakagawa & Yuan, 2000), which is also activated by the buildup of unfolded proteins in the endoplasmic reticulum (ER) under stress conditions (Zhang & Kaufman, 2006). The ER stress response is associated with neurodegeneration, and the ability of PM to penetrate to the brain (Oberdorster et al., 2004) may partially explain PM-associated neuropathology (Peters et al., 2006). The observed caspase-12 activation did not exhibit a strong dose response as measured by Western blot; however, the response was consistently greater in PM-treated cell lysate. Other PM studies that have utilized Western blotting have also shown marked differences from control while not exhibiting a strong dose response (Choi et al., 2004). To our knowledge, caspase-12 has not been previously examined in BEAS-2B cells. While the commercial murine polyclonal differs from those used in other studies, it detected the 25-kD cleavage fragment (that follows cellular stress in lung cells such as respiratory sincitial virus infection) but not the 40-kD fragment (Bitko & Barik, 2001). The $PM_{2.5}$-dependent increase in the 60-kD procaspase-12 as well as its 25-kD cleavage product, in addition to MICA upregulation, seem to support the hypothesis that these particulates induce cellular and ER stress, which may include downstream events such as calpain activation, which are the subject of current investigation. Active caspase-12 is believed to ultimately lead to activation of the effector caspase, caspase-3 (Sun et al., 2000). While increases in the inactive, full-length caspase-3 protein in $PM_{2.5}$-treated cells were detected, as predicted by microarray analysis (Table 1), neither caspase-3 cleavage nor caspase-3 activity was observed. For activity to occur, full-length caspase-3 must be cleaved to active zymogens (Susin et al., 1997). Lack of caspase-3 activation may be explained by the fact that activation of the IL-6 pathway was found to inhibit apoptosis (Hodge et al., 2005) and caspase-3 activation (Chen et al., 1999). The pro-survival signaling of the IL-6 pathway may override the proapoptotic signaling of caspase-12 in these cells.

Cache Valley $PM_{2.5}$ is chemically distinct from that in other Utah urban areas, such as the Salt Lake and Utah Valleys, urban areas characterized by greater population, vehicular traffic, and heavy industry. Particulates and dusts from those locales contain metals that have been the focus of several studies (Wu et al., 2001; Veranth et al., 2004). The iron content of Cache Valley $PM_{2.5}$ (0.82–17.73 nmol/m^3) (Mangelson et al., 1997) is substantially lower than in other studies, where iron was considered the bioactive component of PM (Frampton et al., 1999; Molinelli et al., 2002; Upadhyay et al., 2003). The Cache Valley lacks the steel, smelting, and foundry industries of Utah Valley (Mangelson et al., 1997), and the intensity of motor vehicle use and the desert dust of the Salt Lake Valley (USCB, 2006; Malek et al., 2006; Pope et al., 1999). It is possible that fine particles from these Utah locations have common components, such as bacterial antigens. However, there is conflicting evidence on the importance of bacterial components such as lipopolysaccharide (LPS) in the in vitro toxicity of PM and dusts (Soukup & Becker, 2001; Veranth et al., 2004).

It is important to point out that upregulations of some of the genes detected by microarray in cells exposed to Cache Valley $PM_{2.5}$ (caspase-3, TOLLIP, IL-1R1, and TLR-5) are also recognized cellular responses to bacterial components (Noulin et al., 2005). For example, TLR-5 mediates mammalian defense responses to bacterial flagella (Smith & Ozinsky, 2002). It is hypothesized that bacterial components play a major role in the cellular response to particulate air pollution (Becker et al., 2005). Furthermore, inhibition of CD-14, a receptor protein involved with toll-like receptor proteins, significantly inhibited IL-6 release in alveolar macrophages

(Becker, et al. 2002). Low levels of lipopolysaccharide (LPS) have been shown to induce IL-6 and IL-8 release from BEAS-2B cells (Schulz et al., 2002), despite an internalized TLR-4 LPS receptor, via a mechanism that is not completely understood (Guillot et al., 2004). The quantity of LPS required for IL induction in these cells is unknown. Whether LPS alone or other components are responsible for the observed alteration of basal levels of IL-6 is unknown. While not detected in the $PM_{2.5}$ used in our study, LPS was found in PM_{10} samples from the same Cache Valley locations. LPS has been shown to upregulate expression of IL-1R1 mRNA in mice (Pournajafi Nazarloo et al., 2003) and IL-6R in rats (Vallieres & Rivest, 1999). Interleukin receptor upregulation has been documented following exposure to gram-negative bacteria and may assist in angiogenesis (McCord et al., 2006). Receptor upregulation has also been observed following viral infection and treatment with double-stranded RNA (Zhou et al., 2006; Tsuji et al., 2005) Thus, undetectable bacterial or viral antigens in our $PM_{2.5}$ samples may have been responsible for some of the effects observed in this study.

In the present study, PM concentrations to which the BEAS-2B cells exposed were reasonable approximations of "real-world" conditions. The lowest $PM_{2.5}$ concentrations showing gene-specific effects (0.65 μg/ml, equivalent to 0.08 μg/cm^2 given 75 cm^2 flask area in 10 ml media) were in the same order of magnitude as the theoretical particle deposition (0.01 μg/cm^2) to epithelial cells in vivo resulting from a "high" environmental exposure as postulated by Jimenez et al. (2000). Many of the gene-specific effects of Cache Valley $PM_{2.5}$ were detected at concentrations substantially lower than those in some other studies. This may not be due to inherent differences in particle effects, but to experimental protocols such as our use of larger flasks (75 cm^3) and media volume (10 ml) to facilitate RNA and protein isolation, which resulted in particle dilution. Further, the sensitivity of the assays used with detection limits of <30 copies of specific genes and femtogram levels of protein (qRT-PCR and Western blotting, respectively) revealed effects at low particle concentrations. The observed fold changes in gene expression compared to control were similar to those in other studies using human lung cells exposed to higher particle concentrations (albeit using different RT-PCR protocols). Particle-dependent release of IL-6 seen in our study was comparable to that in BEAS-2B cells exposed to larger concentrations of soil dust particles (Veranth et al., 2004). In comparison with other studies on the effects of Utah PM in BEAS-2B cells, the nitrate rich Cache Valley $PM_{2.5}$ appears more potent in inducing IL-6 release than the calcium rich dust collected from the Utah West Desert (Veranth et al., 2004) or the iron rich Utah Valley particles (Frampton et al., 1999). Such potency differences could have been due to differing culture conditions (plate coatings, media), differences in data presentation, or the presence of bacterial components. The primary sampling site in our study is located 1.6 km from a dairy, and higher ambient endotoxin levels are associated with areas of intensive livestock production relative to urban air (Schulze et al., 2006). In any event, our results clearly demonstrate that low concentrations of urban particulate can elicit measurable and statistically significant responses in vitro.

While caution should be used in attributing environmental relevance to our in vitro data, this initial study may have some value in determining potential adverse health risks associated with exposure to Cache Valley $PM_{2.5}$. The $PM_{2.5}$-induced activation of inflammatory cytokines observed is consistent with the cardiovascular and other diseases associated with exposure to urban PM. This study affirms an increasing body of evidence that IL-1 and IL-6 signaling play a role in the observed health effects of particulate air pollution.

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