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ORGAN TOXICITY AND MECHANISMS

Urban particulate matter activates Akt in human lung cells

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Abstract The normally picturesque Cache Valley in northern Utah is frequently reported to have the worst particulate (PM) air pollution in the United States. Numerous epidemiological studies conducted elsewhere have associated PM exposure to a variety of cardiovascular diseases and early mortality. We have previously shown that Cache Valley PM (CVPM) is pro-inflammatory, through a variety of mechanisms involving the release of inflammatory cytokines, unfolded protein response, ER stress, and C-reactive protein (CRP). This study was undertaken to determine whether Cache Valley PM (CVPM) would activate Akt, an upstream mechanism common to these events. Human lung (BEAS-2B) cells were treated with either fine ($PM_{2.5}$) or coarse (PM_{10}) particles (12.5 and 25 μg/ml) for periods up to 24 h. PM-exposed cells exhibited Akt activation as evidenced by phosphorylation at Thr^{308} and Ser^{473}. Events downstream of Akt activation such as NF-κB activation were observed at 1 and 24 h, but IκB phosphorylation occurred only at 24 h, indicating that mechanisms of PM-mediated NF-κB activation are time dependent. Akt and NF-κB related inflammatory cytokine IL-1α, and IL-6 and the chemokine IL-8 were upregulated in treated cells at 6 and 24 h. The calpain inhibitor leupeptin limited Akt phosphorylation to Ser^{473} and reduced release of IL-1α, IL-6, and IL-8, indicating that calpain or similar protease(s) are involved in PM-induced activation of Akt and subsequent release of inflammatory cytokines. Our data indicate that PM activates Akt, which may play a role in the pro-inflammatory response to PM exposure.

Keywords Akt · Particulate air pollution · $PM_{2.5}$ · LPS · Leupeptin · PTEN · Cytokine

Introduction

The normally picturesque Cache Valley in northern Utah frequently experiences the worst particulate air pollution (PM) in the United States, a situation exacerbated by winter atmospheric inversions that trap and concentrate pollutants (Edgerton et al. 2006; Lurmann et al. 2006; Malek et al. 2006; USA-Today 2005). Similar to PM such as that from the San Joaquin Valley of California, the single largest chemical component of Cache Valley PM (CVPM) is ammonium nitrate (NH_4NO_3), a secondary pollutant formed by atmospheric reactions between nitrogen oxides from vehicles, and ammonia gas from the excreta of dairy cows and other animals (Malek et al. 2006; Mangelson et al. 1997; National Center for Environmental Assessment (Research Triangle Park N.C.) 1996; Edgerton et al. 2006; Lurmann et al. 2006).

In other locations, exposure to PM is associated with early all-cause mortality, as well as to numerous cardiovascular and cardiopulmonary diseases, including ischemic heart disease, cardiac arrest, hypertensive disease, cerebrovascular disease, pneumonia, influenza, in addition to diabetes, neurodegeneration, and cancer (Peters et al. 2006;

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Pope et al. 2002; Pope et al. 2004a). Particulates are chemically diverse and elicit inflammatory and pro-oxidative responses in a variety of cell types through differing mechanisms (Tao et al. 2003). U.S. Federal regulations have been implemented following epidemiological studies regulating 2 PM size fractions of which $PM_{2.5}$ appeared to have greater association with adverse health effects than PM_{10} (Schwartz et al. 1996; Delfino et al. 2005), although some other studies indicate a significant health threat posed by the larger PM_{10} (Choi et al. 2004; Osornio-Vargas et al. 2003; Pozzi et al. 2003; Monn and Becker 1999; Soukup and Becker 2001; Jalava et al. 2007; Becker et al. 2005c; Becker and Soukup 2003).

While little is known about the mechanisms by which PM may induce harm (Lippmann et al. 2003; Mossman et al. 2007; NHEERL 2010; National Center for Environmental Assessment (Research Triangle Park N.C.) 2003), the inflammatory response has received the most attention (Delfino et al. 2005; Pope et al. 2004b; Donaldson et al. 2001; Schins et al. 2004). A potential mechanism for the pro-inflammatory action of PM is through activation of nuclear factor-kappa B (NF-κB), a transcription factor stimulated in response to many pro-inflammatory agents (Jimenez et al. 2000; Dagher et al. 2007), which is activated by Akt (formerly known as protein kinase B). Another important component of inflammation is apoptosis suppression in cytokine-releasing cells, a process that also involves Akt (Abraham 2005).

Akt is a cytosolic serine/threonine kinase (Rane et al. 2003) required for cancer cell growth, promoting the survival of cancer cells exposed to chemotherapy and radiation-induced apoptosis (Brognard et al. 2001). Activated Akt is detectible in 90% of non-small cell lung cancer cell lines, and 100% of those derived from smokers (West et al. 2003). Akt contributes to lung inflammation and injury (Abraham 2003), promotes chemotaxis to fight bacterial infection (Abraham 2005), and is a pro-survival adaptation mechanism following stress (Hu et al. 2004). Akt contributes to inflammation activating the pro-inflammatory transcription factor NF-κB playing a central role in survival by altering cellular metabolism and inhibiting apoptosis (Venkatachalam et al. 2008; Newcomb et al. 2008; Amaravadi and Thompson 2005; Chen 2005; Misra et al. 2006). Akt regulation is primarily through posttranslational modification (Amaravadi and Thompson 2005). Prototypical Akt activation occurs though receptor-mediated activation of phosphatidylinositol 3-kinase (PI3K). Activated PI3K produces 3-phosphoinositides (PIP_3), which bind to and induce a conformational change in Akt as well its translocation from the cytoplasm to the plasma membrane where it is phosphorylated. Akt is fully activated when phosphorylated at Ser^{473} and Thr^{308}, a process inhibited by tumor suppressor phosphatase and tensin homolog (PTEN) that dephosphorylates PIP_3 (Cantley and Neel 1999). PTEN also limits the action of MDM2 allowing upregulation of p53 activity (Brognard et al. 2001; Hajduch et al. 2001). PTEN is inactivated by phosphorylation of its C-terminal tail (Vazquez et al. 2000), an event associated with a number of human tumors (Meek and Knippschild 2003).

We have previously demonstrated that ambient CVPM activates Stat3, P70S6 kinase, calpain, and Hsp27, and concomitant increases in Hsp90 protein in human airway epithelial (BEAS-2B) cells (Watterson et al. 2007). Because these events are associated with Akt (Watterson et al. 2007; Rane et al. 2003; Chatterjee et al. 2006; Tan et al. 2006; Peterson and Schreiber 1998; Watterson et al. 2009a, 2009b), we hypothesized that CVPM would activate Akt and related proteins leading to the release of Akt-associated inflammatory cytokines.

Materials and methods

Chemicals and reagents

BEAS-2B cells were a kind gift from Dr. Katerine Macé (Nestle Research Centre; Lausanne, Switzerland). Recombinant IL-1 was from R&D Systems (Minneapolis, MN). LHC-9 cell growth media was from Invitrogen (Camarillo, CA). IRAK-1 primary antibody was from Affinity Bioreagents (Golden, CO). All remaining antibodies, CHAPs cell extract buffer, and biotinylated molecular weight ladders were from Cell Signaling Technology (Danvers, MA). The Akt antibodies were not isoform specific but were able to detect total Akt or phosphorylated total Akt. Cytokine antibody array was from Quansys Biosciences (Logan, UT). Lipopolysaccharide (LPS) from *Salmonella minnesota* was from Alexis Biochemicals (San Diego, CA). Restore Western Blot Stripping buffer was from Pierce (Rockford, IL). CellBIND 6-well cell culture plates were from Corning (Corning, NY). Nitrocellulose membranes were from Bio-Rad (Hercules, CA).

PM collection and extraction, Cell culture, and exposure

PM sampling, extraction, endotoxin detection, and culture of BEAS-2B cells were previously described (Watterson et al. 2007; Watterson et al. 2009a, b). For time course studies, cells from the same passage were seeded onto separate plates and cell numbers adjusted accordingly (approximately 3.6×10^2 cells/well). Cells were grown until ~80% confluent then treated with fine ($PM_{2.5}$) and coarse (PM_{10}) at "low" (12.5) or "high" (25 μg/ml) concentrations. These concentrations are below a hypothetical "high" exposure of 50 μg/ml (Becker et al. 2005b) and

represent potential "real-world" exposures. In all experiments, LPS (10 ng/ml) was also used for comparison. None of the concentrations of PM or LPS used in this study are cytotoxic to BEAS-2B cells (Watterson et al. 2009a, 2009b). Because Akt activation can occur rapidly (West et al. 2003), cells were harvested at 1 and 24 h time points with cytokine release examined at 1, 6, and 24 h.

Western blotting and ELISA

Methods for isolation of cell lysates, western blotting, and luminescent analysis have been previously described in detail (Van Vleet et al. 2006; Watterson et al. 2007) with the exception that PVDF membranes were used in this study rather than nitrocellulose. As PVDF allows for stripping and re-probing with additional primary antibodies, where appropriate, PVDF membranes were stripped for 15 min at room temperature. Membranes were re-incubated in chemiluminescent substrate for 5 min and reexamined to ensure proper stripping. Membranes were then re-probed with primary antibodies, and images were captured using an imaging workstation (UV Products, Upland, CA), and analysis was performed using the histogram function in Adobe PhotoShop CS (San Jose, CA) (Woznicova and Votava 2001). The software provided mean pixel intensity, standard deviations, and total number of pixels sufficient for one-way analysis of variance by Holm Sidak with significance set at $p < 0.05$. Mean luminescent values of the selected bands obtained from Adobe PhotoShop (San José, CA) were normalized to control and to bands of unmodified protein or to β-actin on the same membrane. Gels were loaded with 10 μg protein/well. Media from treatments was saved for ELISA, which was performed on multiplex chemiluminescent ELISA plates (Quansys Biosciences, Logan, UT) according to the manufacturers' instructions as previously described (Barnard et al. 2006; Yuan et al. 2007; Gowen et al. 2006). Curve fitting and analysis were performed using SigmaPlot with the curve presenting the lowest PRESS statistic judged to have the best fit for interpolation (Daly et al. 2005).

Statistical analysis

One-way analysis of variance (ANOVA) was performed to examine differences between 3 or more groups, with *post hoc* Holm–Sidak multiple comparisons analysis (Glantz 2005). Where type II error was suspected from multiple comparisons, the "versus control" function of the Holm–Sidak analysis was utilized. Comparisons between two groups were performed using the t-test. SigmaStat software (SYSTAT, San José, CA) was used for all testing. $P \leq 0.05$ was judged to be significant.

Results

Akt requires phosphorylation at Thr^{308} and Ser^{473} for full activation, which was visualized on immunoblots. Akt was constitutively active (Fig. 1a), and cells exposed to PM had significant increases in Akt phosphorylation at both Ser^{473} and Thr^{308}, although phosphorylation at Thr^{308} appeared to be affected more than Ser^{473} (Fig. 1). After 1 h, only low concentrations of both PM size classes provoked significant Akt phosphorylation ($P \leq 0.001$ for both Thr^{308} and Ser^{473}) compared to control (Fig. 1a). However, at 24 h, Akt phosphorylation at both Thr^{308} and Ser^{473} was significantly ($P < 0.001$) elevated in lysates from all PM treatments compared to control and to LPS-treated cells (Fig. 1b).

Further evidence of Akt activation was determined by observing the effects of PM treatment on two upstream proteins responsible for mediating Akt phosphorylation: phosphoinositide-dependent protein kinase 1 (PDK-1), which phosphorylates Akt at Thr^{308} (Williams et al. 2000) while in the process is phosphorylated itself at Ser^{241} (Casamayor et al. 1999), and PTEN, a negative effector of Akt phosphorylation. Other proteins affected by activated Akt, glycogen synthase kinase (GSK) that is inactivated by Akt via phosphorylation at Ser^{9}, and p53 whose levels are decreased by activated Akt (Limesand et al. 2006) were also examined. After either 1- (Fig. 2) or 24-h (Fig. 3) exposures to fine and coarse PM, there was heightened PDK phosphorylation compared to control and LPS ($P \leq 0.001$), with the greatest responses at 1 h (Fig. 2) seen in cells exposed to low $PM_{2.5}$ and nearly equivalent responses with the fine PM after 24 (Fig. 3, $P = 0.242$). At 1 h, the signal for p-PTEN was detectable but too faint to reliably analyze (Fig. 2), but significant ($P \leq 0.001$) increases in this protein were observed after 24-h exposures compared to control (Fig. 3). The greatest response for p-PTEN at 24 h was with the high concentration of coarse PM and LPS, which were nearly identical ($P = 0.5$; Fig. 3).

Appearance of the inactivated p-GSK was increased in $PM_{2.5}$, but decreased in cells exposed to PM_{10} relative to control ($P \leq 0.001$) following 1 h (Fig. 2). After 24-h treatments, p-GSK declined to levels below that of control (Fig. 3).

Western blots of p53 showed PM-related increases in p53 from cell lysates exposed to both size fractions of PM compared to control after 1-h exposure ($P \leq 0.001$) (Fig. 2). Low $PM_{2.5}$ caused the greatest increase in p53, which was significantly greater than the high $PM_{2.5}$ and both concentrations of PM_{10} ($P \leq 0.001$). There was no significant difference between the two PM_{10} concentrations ($P = 0.405$) or from the high $PM_{2.5}$ to either low or high PM_{10} ($P = 0.455$ and 0.169, respectively). At 1 h, the

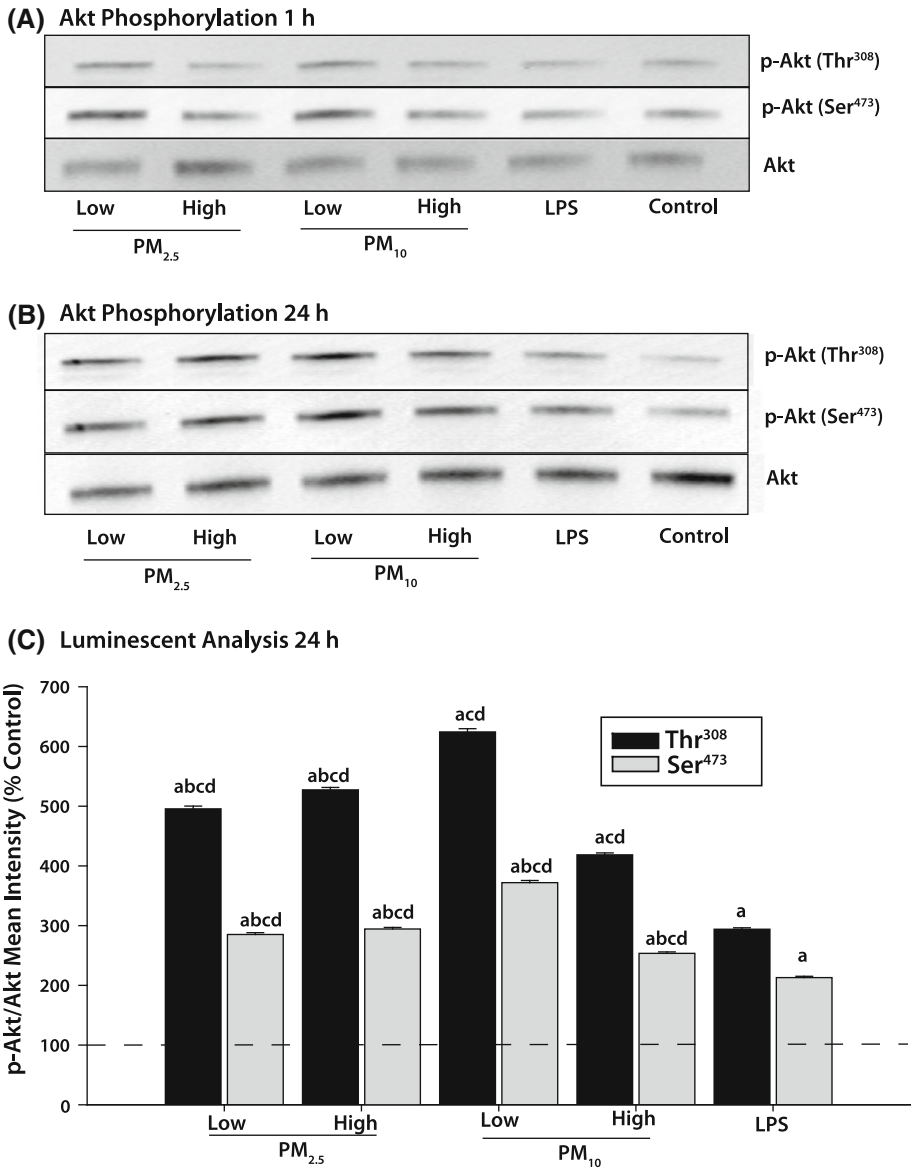

Fig. 1 PM treatment resulted in marked increases in Akt phosphorylation in BEAS-2B cells. Western immunoblots (**a**–**b**) and luminescent analysis (**c**) examining the activation state of Akt (60 kDa). Blots are of cell lysates treated with 1- (**a**) or 24-h (**b**) exposures of 12.5 (low) and 25 (high) μg/ml $PM_{2.5}$ and PM_{10} including 10 ng/ml LPS and untreated negative control. Gels were loaded with 10 μg protein. Figures are representative of a minimum of 3 independent experiments. Results are presented as mean intensity normalized to control and unmodified Akt ± SE. *a* significant from control, *b* significant from differing concentration of the same PM diameter, *c* significant from same concentration of differing PM diameter, *d* significant from LPS ($P \leq 0.05$)

signal for the second of the doublet bands often seen by this particular antibody in lysates from BEAS-2B cells (Van Vleet et al. 2006) was visible but not measureable as shown in Fig. 2a. Following 24 h, p53 was observed as doublet bands with no significant alterations in treatment-mediated response ($P = 0.97$, luminescent analysis not shown Fig. 3b). The signal responses (212–238 luminescent pixels) were below that of saturation (246 luminescent pixels), indicating that the equivalent p53 response was not due to image overexposure. The lower band in the doublet, likely indicating a different phosphorylation state (Van Vleet et al. 2006), was also examined with no significant differences between groups ($P = 0.067$, luminescent analysis not shown). Akt-mediated phosphorylation of PDK endothelial nitric oxide synthase (eNOS) on the Ser^{1177} site was examined but not detected following 1- and 24-h exposures (data not shown).

Given the documented ability of Akt to activate NF-κB (Misra et al. 2006), alterations in NFκ-B were examined as a marker of Akt activation. NF-κB is classically activated by phosphorylation and subsequent degradation of the inhibitory protein IκB, although degradation can also be mediated through calpain (Pianetti et al. 2001). Alternative NF-κB activity is through the constitutive processing of p105 to p50 and the inducible processing of p100 to give p52 (Hayden and Ghosh 2004). Therefore, both pathways of NF-κB activation were explored in PM-exposed cells.

Expression of constitutive IκB was clearly evident in all PM-treated cells after either 1- (Fig. 4) or 24-h (Fig. 5) exposures; 1-h exposures to PM did not result in the appearance of p-IκB, a measure of classical NF-κB activity (Fig. 4). Levels of inactive NF-κB p100 were slightly increased from control ($P = 0.018$ for the high $PM_{2.5}$, $P \leq 0.001$, for the remainder), with PM_{10}-exposed cells

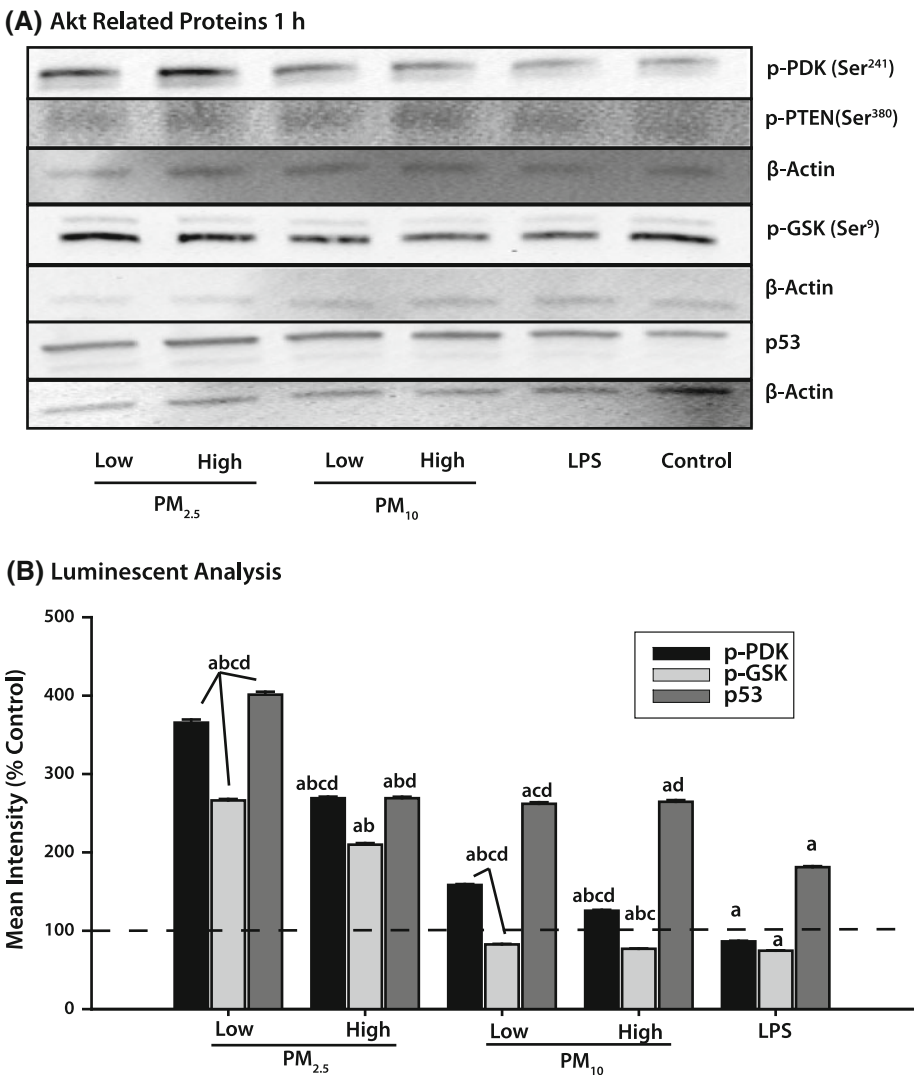

Fig. 2 PM treatment induced alterations of Akt-related proteins in BEAS-2B cells following 1-h exposure. Western immunoblots (**a**) and luminescent analysis (**b**) examining the alterations of the phosphorylation states of PDK (58–68 kDa), PTEN (54 kDa), and GSK (46 kDa), and the total levels of p53. Gels were loaded with 10 μg protein. Blots are of lysates from cells receiving 1-h exposure of 12.5 (low) and 25 (high) μg/ml $PM_{2.5}$ and PM_{10} including a 10 ng/ml LPS-positive control and untreated negative control. Figures are representative of a minimum of 3 independent experiments. Results presented as mean intensity normalized to control and to β-actin (45 kDa) ±SE. *a* significant from control, *b* significant from differing concentration of the same PM diameter, *c* significant from same concentration of differing PM diameter, *d* significant from LPS ($P \leq 0.05$)

giving the greatest response. There was elevated expression of active p52, p50, and p105 ($P \leq 0.001$), in PM-exposed cells compared to control (Fig. 4).

At 24 h (Fig. 5), constitutive p-IκB (Ser^{32}) was detected in all cell lysates with significantly elevated levels in lysates from cells that were treated with PM and LPS ($P \leq 0.001$). Cells treated with PM_{10} had the highest levels of p-IκB, but no concentration effect was observed ($P = 0.589$; Fig. 5). Interestingly, phosphorylation of IκB was not reflected in reductions of total IκB with only the lysates from LPS-treated cells having significantly ($P = 0.031$) lower levels of the protein than control. Lysates from cells treated with the lowest concentration of $PM_{2.5}$ had the highest levels of protein (Fig. 5). With the inducible active p52 protein, lysates from cells treated with PM demonstrated significant increases ($P \leq 0.001$) from control with the exception of the high $PM_{2.5}$ ($P = 0.43$). The LPS-exposed cells expressed lower ($P \leq 0.001$) levels of p52 than that from control (Fig. 5). While the inactive p100 dimer was not detectable, there was increased expression of the inactive p105 dimer in PM-treated cells ($P \leq 0.001$ for all) compared to control. All PM treatments induced increases ($P \leq 0.001$) in the active p50 protein.

Cytokine release is regulated by activities of Akt (Strassheim et al. 2004) and NF-κB (Hayden and Ghosh 2004), so the ability of CVPM to induce cytokine release was examined. IL-1α, IL-6, and IL-8 were detected, and IL-1β, IL-2, IL-4, IL-10, interferon-γ, TNF-α, and TNF-β were not. Cytokine release was detectable but not quantifiable 1 h posttreatment but was measurable after 6 h (Fig. 6). At that time point, the fine ($P \leq 0.004$) but not the coarse ($P \geq 0.128$) elicited significantly ($P = 0.00$) greater IL-1α release than control. At 24 h, only the low-fine exhibited significantly ($P = 0.002$) greater IL-1α release compared to control (Fig. 6). With IL-6 following 6-h exposures, all treatments ($P \leq 0.005$) excepting the low-coarse ($P = 0.174$) induced significantly greater IL-6 levels than control with LPS eliciting a 4× greater response

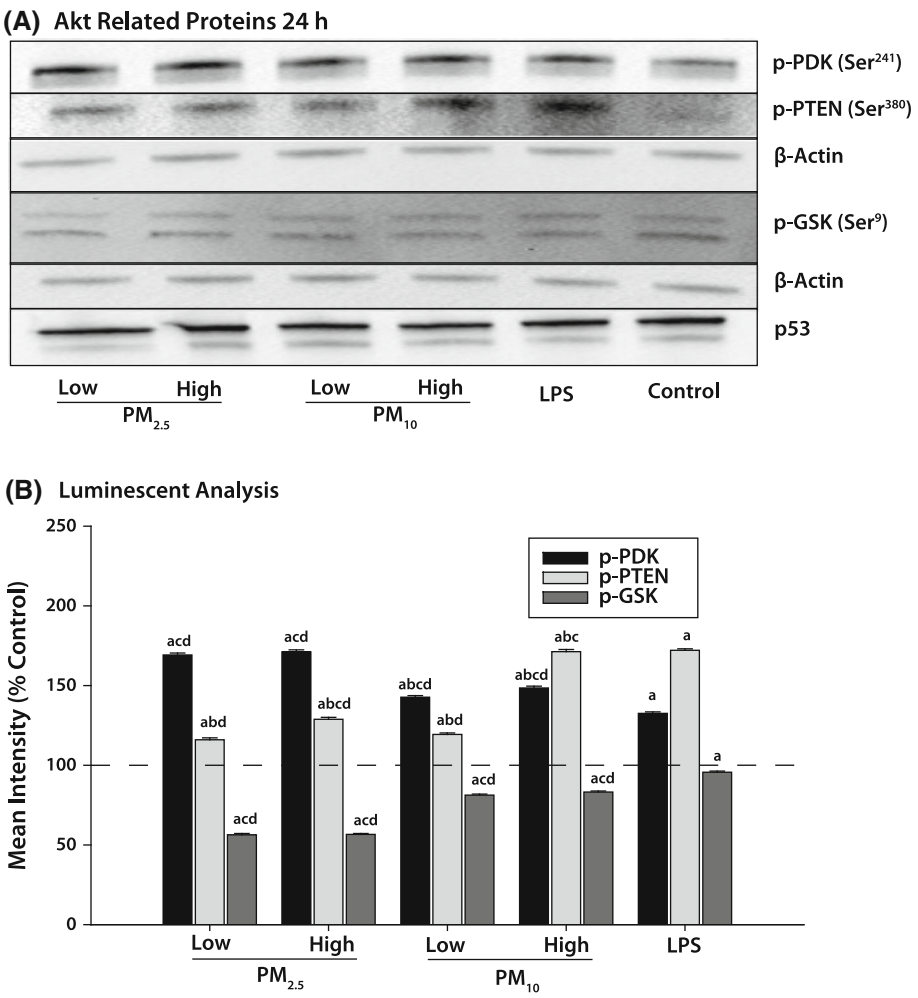

Fig. 3 PM treatment induced alterations of Akt-related proteins in BEAS-2B cells following 24-h exposure. Western immunoblots (**a**) and luminescent analysis (**b**) examining the alterations of the phosphorylation states of PDK (58–68 kDa), PTEN (54 kDa), and GSK (46 kDa), and the total levels of p53. Blots are of lysates from cells receiving 24-h exposures of 12.5 (low) and 25 (high) μg/ml $PM_{2.5}$ and PM_{10} including a 10 ng/ml LPS-positive control and untreated negative control. Gels were loaded with 10 μg protein. Figures are representative of a minimum of 3 independent experiments. Results presented as mean intensity normalized to control and to β-actin (45 kDa) ± SE. *a* Significant from control, *b* significant from differing concentration of the same PM diameter, *c* significant from same concentration of differing PM diameter, *d* significant from LPS at $P \leq 0.05$

than that of the PM. Following 24 h, all treatments exhibited significant increases from control ($P \leq 0.001$) with LPS inducing only a 40% greater response than the nearest PM-induced response (Fig. 6). IL-8 release was not significantly affected by PM treatment after 6 h, and only the high $PM_{2.5}$ elicited (and LPS) a greater response than control after 24 h ($P \leq 0.001$ each).

Previous studies have shown that CVPM treatment results in increases in extracellular Hsp70 (Watterson et al. 2009a, b), which exhibits its signaling capacity through the toll-like receptor (TLR)/IL-1 receptor pathway (Vabulas et al. 2002). As there were PM-mediated IL-1 release and alterations in NF-κB activity, which can also be activated through IL-1R1 activation, we examined the central regulator of TLR-2,4/IL-1R the interleukin-1 receptor-associated kinase 1 (IRAK) (Arcaroli et al. 2006). IRAK phosphorylation was not detected on Thr^{387}, which is required for IRAK activation (Kollewe et al. 2004), following 1- or 24-h of PM and LPS exposure (Fig. 7). IRAK phosphorylation at Ser^{376}, which is indicative of IRAK-4 activation (Koziczak-Holbro et al. 2007) likewise, was not detected (data not shown). Inactive IRAK was detected and was increased in PM-treated cells, which is consistent with PM treatment (Watterson et al. 2007) and indicative of a lack of IRAK degradation (Yamin and Miller 1997). Antibody functionality was verified by treating cells with 25 ng/ml IL-1α, which caused IRAK phosphorylation at both Ser^{376} and Thr^{387} sites following 1- and 24-h treatment (data not shown).

In order to determine whether calpain activation might be involved in PM-mediated Akt phosphorylation (Tan et al. 2006), cells were co-incubated with a non-cytotoxic concentration of 40 μg/ml leupeptin (Momiyama et al. 2006), a "relatively select" calpain inhibitor (Takahashi et al. 2006) for 24 h. Leupeptin is water soluble so does not need to be used with a vehicle that might itself alter some of the subtle pathways that PM alters such as PEG (Ono et al. 1999), ethanol (Carloni et al. 2004), and DMSO (our laboratory, unpublished findings) altering calpain activity. Leupeptin eliminated detectable Akt phosphorylation at Thr^{308}, PTEN phosphorylation, PDK phosphorylation, and Gsk phosphorylation (data not shown). Leupeptin

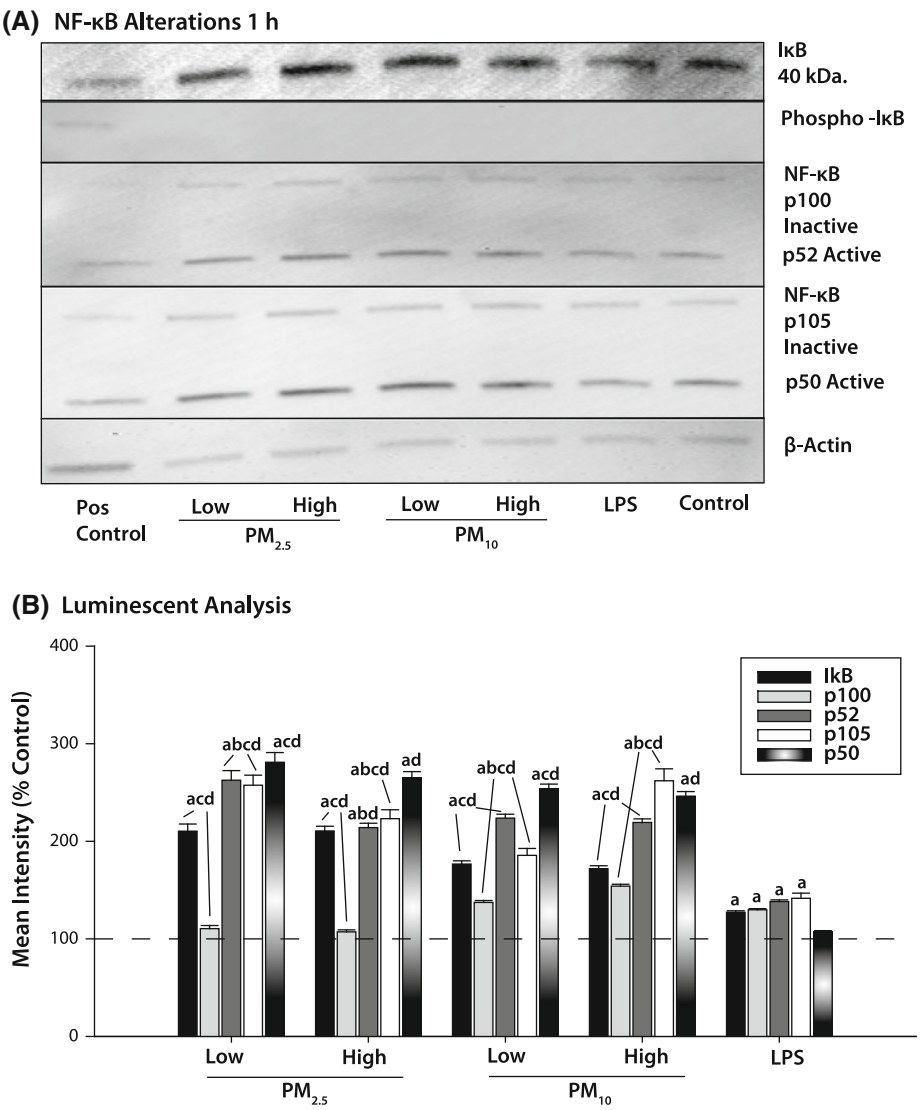

Fig. 4 PM alters non-classical NF-κB activity in BEAS-2B cells after 1-h incubation. Western immunoblots (**a**) and luminescent analysis (**b**) examining alterations of IκB (40 kDa), NF-κB p100/52, and NF-κB p105/p50. Gels were loaded with 10 μg protein/well with the exception of the positive control lane which was a lysate of TNF-α treated HeLa cells provided by the antibody manufacturer loaded at 10 μl/well. Blots are of lysates from cells receiving 1-h exposures of 12.5 (low) and 25 (high) μg/ml $PM_{2.5}$ and PM_{10} including a 10 ng/ml LPS-positive control and untreated negative control. Figures are representative of a minimum of 3 independent experiments. Results presented as mean intensity normalized to control and to β-actin (45 kDa) ± SEM. *a* Significant from control, *b* significant from differing concentration of the same PM diameter, *c* significant from same concentration of differing PM diameter *d* significant from LPS at $P \leq 0.05$

co-treatment had little effect upon the detection of Akt phosphorylation at Ser^{473}. Leupeptin co-incubations with PM and LPS exhibited significantly greater effects than leupeptin alone control ($P \leq 0.001$) except with the low concentration of the fine ($P = 0.296$) PM (Fig. 8a). Cytokine levels were examined in the conditioned media from the leupeptin plus PM and LPS treatments. For IL-1α, the high $PM_{2.5}$ and both low and high PM_{10} plus leupeptin treatments achieved significantly different results from the leupeptin only ($P = 0.004$, $P = 0.001$, and $P = 0.001$, respectively). Leupeptin plus the low concentration of fine PM and leupeptin plus LPS were not significant from leupeptin only ($P = 0.140$, and $P = 0.09$). In the case of IL-6, the leupeptin co-treatment eliminated the PM-mediated increase from control of the $PM_{2.5}$ and the low concentration of the PM_{10}. Only the high (25 μg/ml) and LPS treatments exhibited significantly greater effects than control ($P = 0.001$ and $P \leq 0.001$, respectively). Cells co-treated with both types of PM and leupeptin had slightly less IL-8 release than the cells treated with leupeptin only. This reduction was not significant ($P = 0.067$ and $P = 0.122$ for low and high $PM_{2.5}$-treated cells and $P = 0.252$ and $P = 0.077$ for low and high PM_{10}-treated cells). Only the LPS plus leupeptin co-treatment group achieved a significantly greater response ($P \leq 0.001$) from the leupeptin-only group (Fig. 8b).

Discussion

To our knowledge, this study is the first to demonstrate that ambient PM activates Akt in vitro with concomitant inactivation of the tumor suppressor PTEN. Previous studies have shown that reserve oil fly ash altered related Akt genes in rat neonatal cardiomyocytes (Knuckles and Dreher 2007) and diesel exhaust particles (DEP) induced

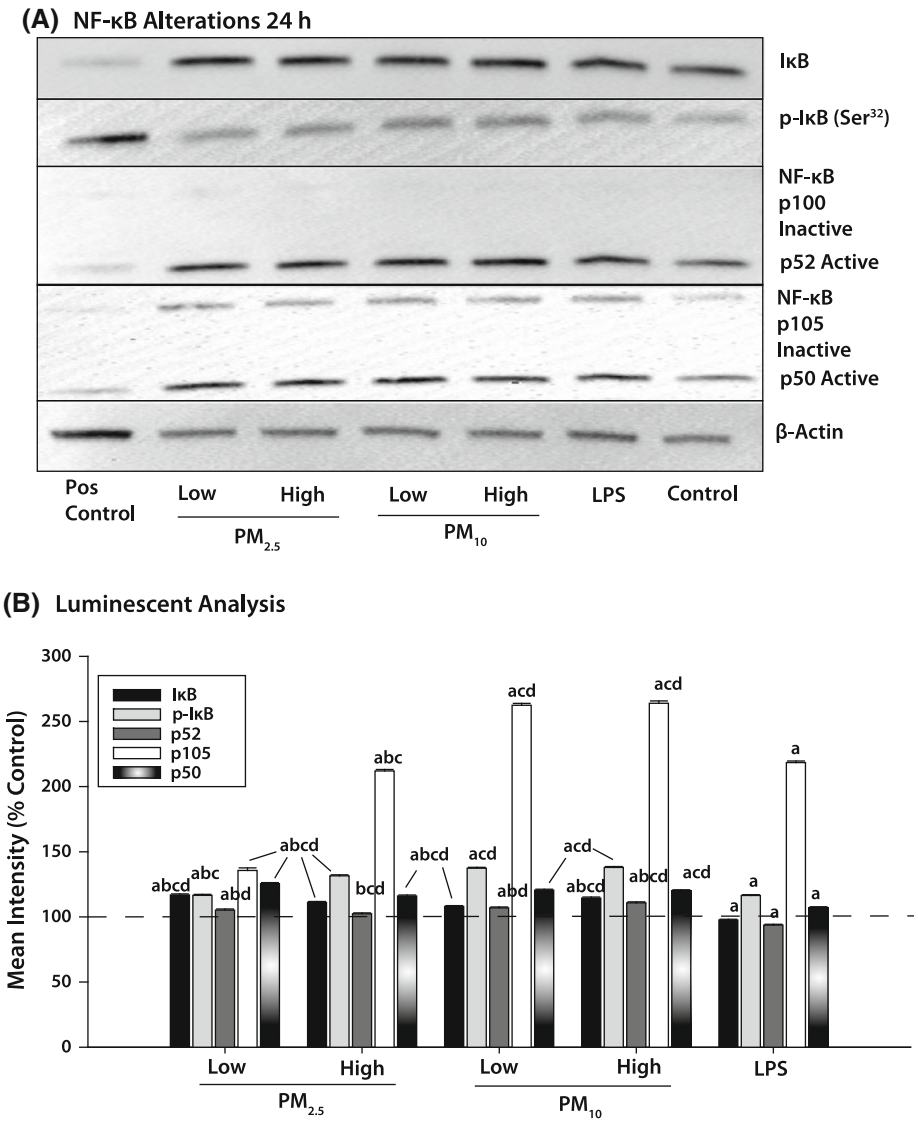

Fig. 5 PM alters classical NF-κB activity in BEAS-2B cells after 24-h incubation. Western immunoblots (**a**) and luminescent analysis (**b**) examining alterations of IκB (40 kDa), NF-κB p100/52, and NF-κB p105/p50. Gels were loaded with 10 μg protein/well with the exception of the positive control lane which was a lysate of TNF-α-treated HeLa cells provided by the antibody manufacturer loaded at 10 μl/well. Blots are of lysates from cells receiving 24-h exposures of 12.5 (low) and 25 (high) μg/ml $PM_{2.5}$ and PM_{10} including a 10 ng/ml LPS-positive control and untreated negative control. Figures are representative of a minimum of 3 independent experiments. Results presented as mean intensity normalized to control and to β-actin (45 kDa) ± SEM. *a* Significant from control, *b* significant from differing concentration of the same PM diameter, *c* significant from same concentration of differing PM diameter, *d* significant from LPS at $P \leq 0.05$

Akt activity in human umbilical vein epithelial cells (Sumanasekera et al. 2007). In murine keratinocytes, DEP activated Akt in the absence of PTEN phosphorylation (Ma et al. 2004). In another study, DEP (50 μg/ml) caused downregulation of Akt phosphorylation and concomitant apoptosis in A549 cells, events that were suppressed when these cells were stably transfected with recombinant human thioredoxin-1 (*rh*-Trx-1), suggesting a protective role of this protein against reactive oxygen species released from DEP (Kaimul Ahsan et al. 2005). Diet-induced obese mice exposed to PM (1.6 mg/kg, intratracheal instillation and inhalation of 13 $\mu g/m^3$ for 6 h/day, 5 days/week, for 128 days) showed reductions in Akt phosphorylation (Ser^{473}) in intact aorta but no changes in Akt phosphorylation in epithelium-denuded aortic tissues (Sun et al. 2009). Akt directly controls cellular metabolism via hexokinase and phosphofructokinase-2 and is involved in respiratory burst in neutrophils (Chen et al. 2003) likely explaining previous findings of PM-mediated oxidative burst (Soukup et al. 2000) and PM-mediated increases in MTT reduction (Watterson et al. 2007; Pruett and Loftis 1990).

The pro-inflammatory activity of Akt is primarily due to its ability to suppress apoptosis in cytokine-releasing cells (Matute-Bello et al. 1997), a mechanism thought to contribute to acute lung injury (Abraham 2003). Akt suppresses apoptosis through mechanisms involving MDM2 activation and p53 degradation, inactivation of caspase-9 and Bad, stabilization of the anti-apoptotic protein XIAP, Bim downregulation (Amaravadi and Thompson 2005), prevention of mitochondrial cytochrome-c release (Abraham 2003), and forkhead inactivation (Brunet et al. 1999). Akt activation is also a pro-survival compensatory action taken by cells following insult, such as endoplasmic reticulum stress (Hu et al. 2004), ischemia (Mullonkal and Toledo-Pereyra 2007), sepsis, (Li et al. 2004), or ultraviolet

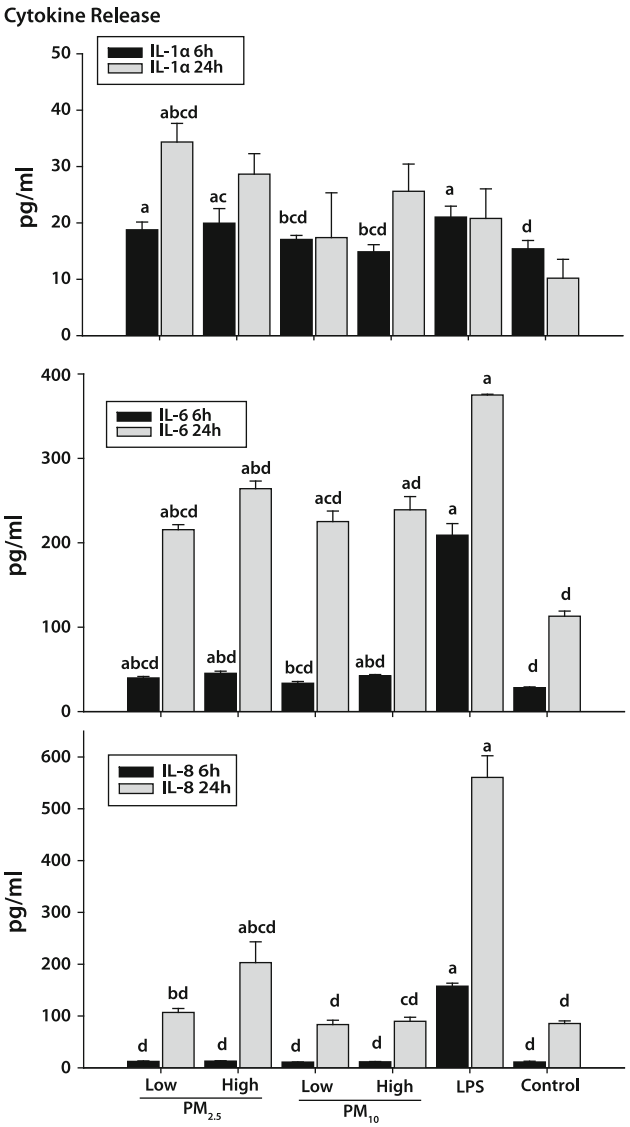

Fig. 6 PM exposure causes the release of NF-κB-related interleukins following 6- and 24-h exposures. Results are a combination of $n = 3$ independent experiments with six replicates and are given in mean concentration (pg/ml) ± SEM. *a* Significant from control, *b* significant from differing concentration of the same PM diameter, *c* significant from same concentration of differing PM diameter, *d* significant from LPS at $P \leq 0.05$

light (Mallikarjuna et al. 2004). As we have shown CVPM to trigger ER stress in these cells (Watterson et al. 2009), it is possible that the Akt activation observed here is also a compensatory action.

The signals for NF-κB activation include Akt, TNF, and TLR network (Hazeki et al. 2007; Oda and Kitano 2006). NF-κB activation typically occurs through the phosphorylation and ubiquitin-mediated degradation of the inhibiting protein IκB-α although NF-κB activation via oxidative stress can bypass IκB-α degradation (Pianetti et al. 2001). PM has been demonstrated to activate NF-κB upon contact with cell surfaces via mechanisms that are independent of IκB degradation (Churg et al. 2005; Jimenez et al. 2000). The lack of IκB degradation with concurrent phosphorylation found in this study may be due to inhibition of proteosomal degradation, or to IκB upregulation, a recovery effect observed following LPS stimulation (Velasco et al. 1997). PM-mediated enhancement of IκB-α phosphorylation observed in the present study is possibly associated with Akt (Abraham 2005) or via calpain (Watterson et al. 2009a, 2009b; Pianetti et al. 2001) which activates Akt (Tan et al. 2006) and can be activated by PM (Watterson et al. 2009). The PM-induced appearance of processed p50 (NF-κB1) and p52 (NF-κB2) fragments is consistent with constitutive NF-κB signaling (Senftleben et al. 2001) and increases in the constitutive activation of Stat3 with PM exposure we previously observed (Watterson et al. 2007; Nadiminty et al. 2006). Of interest are the increases in NF-κB p105/p50, an effect observed with cell migration and tumor progression (Gao et al. 2006).

Akt signaling elicits inflammatory cytokine release (Wong et al. 2007) and is likely to be partially responsible for the release of inflammatory cytokines IL-6 and the chemokine IL-8 observed in this study, in agreement with previous studies using this cell type (Veranth et al. 2004; Frampton et al. 1999).

Chronic PM-mediated IL-6 release may lead to atherogenesis via eNOS inhibition (Saura et al. 2006) and may be a possible mechanism for linking PM to cardiovascular mortality (Pope et al. 2004a) although it may require consistent PM exposure that may or may not occur with ambient conditions. IL-6 is constitutively released in these cells and has potential autocrine signaling, its release is enhanced by PM exposure (Watterson et al. 2007), and eNOS is expressed in the lung and lung cells (Higashimoto et al. 2005; Ten Broeke et al. 2006). Our inability to detect eNOS phosphorylation at Ser^{1177} (Morrow et al. 2003) despite Akt activation, which activates eNOS (Ndiaye et al. 2005), supports these hypotheses of IL-6 inhibiting eNOS and warrants additional study. IL-8 is angiogenic contributing to tumor progression in lung cancer (Yuan et al. 2005; Luppi et al. 2007), but IL-8 autocrine signaling is unlikely here, as evidenced by a lack of observable STAT-1 phosphorylation examined previously (Watterson et al. 2009a, 2009b). While this is not the first study to detect IL-1α release from BEAS-2B cells (Griego et al. 2000), it appears the first to detect PM-mediated IL-1α release from BEAS-2B cells. The contribution of IL-1 to atherogenesis is well documented (Chi et al. 2004), and IL-1 contributes to tumor progression (Lewis et al. 2006; Elaraj et al. 2006). Of the cytokines not detected in this study, TNF-α release from BEAS-2B cells has been documented following exposure to studded-tire wear particles (Lindbom et al. 2006).

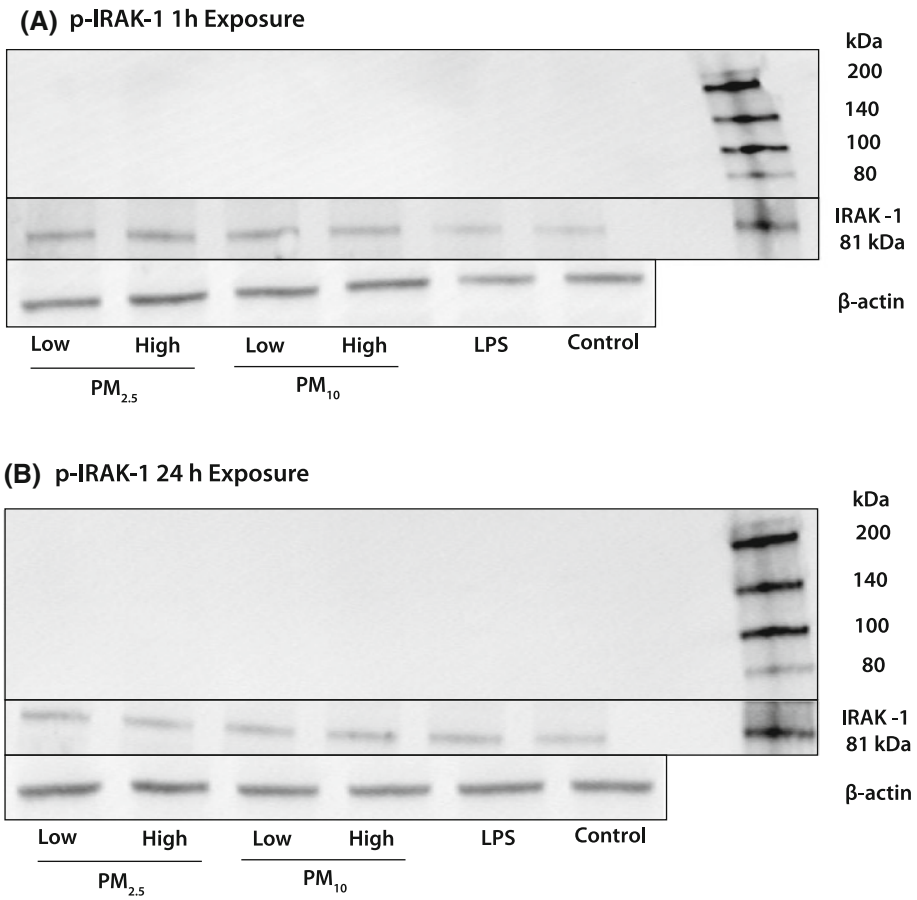

Fig. 7 PM signaling in BEAS-2B cells is not through an IRAK-1-dependent mechanism. Immunoblots revealed that IRAK-1 is not phosphorylated following treatment after 1- (**a**) or 24-h (**b**) treatment. This indicates that the IL-1 receptor pathway is likely not activated by the PM used in this study in BEAS-2B cells. This is despite PM-mediated increases in IRAK levels. Western immunoblots of cell lysates treated with 1- or 24-h exposures of 12.5 (low) and 25 (high) μg/ml Cache Valley $PM_{2.5}$ and PM_{10} including a 10 ng/ml LPS-positive control and untreated negative control. Gels were loaded with 10 μg/ml total protein. Figures are representative of a minimum of 3 independent experiments

This evidence of increases in Akt activation raises additional questions concerning the mechanisms of PM-induced pathogenesis. Akt is well known for its role in insulin signaling (Alessi et al. 1996), and insulin in the growth media (approximately 5 mg/L, Invitrogen Technical support) may be responsible for the basal levels of Akt phosphorylation observed here, as well as in other studies (Zhang et al. 2006). It would be interesting to see whether PM-mediated Akt activation might play a role in the observed association between PM exposure and diabetes prevalence (Pearson et al. 2010). The role of the TLR network in PM-mediated effects has been examined (Becker et al. 2002, 2005a), and the TLR network is implicated in Akt activation (Hazeki et al. 2007; Ha et al. 2008; Li et al. 2004). The current study has demonstrated that PM-mediated Akt and NF-κB activation are through an IL-1R1-independent mechanism as evidenced by the lack of PM-inducible IRAK-1 phosphorylation. This is intriguing given that PM treatment resulted in increases in IRAK-1 protein levels (Fig. 7). TOLLIP may be upregulated resulting in IRAK/MyD88 inhibition, or MyD88 may be activated by other receptors (Hayden and Ghosh 2004). IRAK/dependent signaling represents a small portion of the large TLR signaling network, and many other potential mechanisms of receptor-mediated activation of Akt exist (Oda and Kitano 2006). Akt activation also occurs through receptor tyrosine kinases (RTKs) (Zhang et al. 2007), IL-6 (Chen et al. 1999), the janus kinase (Jak)/Stat pathway (Gross et al. 2006), G-protein-coupled receptors (Kong et al. 2006), peroxisome proliferator–activating receptors, (Amaravadi and Thompson 2005), and calpain (Tan et al. 2006; Pianetti et al. 2001). The leupeptin co-administration experiments demonstrated that the PM-mediated Akt activation is possibly due to calpain or a similar serine/cysteine protease(s). Leupeptin-mediated calpain inhibition appeared to inhibit PDK-1, which phosphorylates Akt at Thr^{308} (Vanhaesebroeck and Alessi 2000) or promoted its dephosphorylation (Gao et al. 2005), while not affecting PM-mediated increases in integrin-linked kinase (Persad et al. 2001), MTOR (Sarbassov et al. 2005), or other unknown protein(s) with PDK-2 activity that phosphorylate Akt at Ser^{473} (Vanhaesebroeck and Alessi 2000). The PM-mediated calpain activation and subsequent Akt activation are possibly due to PM-induced ER stress (Watterson et al. 2009).

There were no consistent differences between the responses to fine and coarse PM in this study. The slight differences observed, when present, were possibly due to a

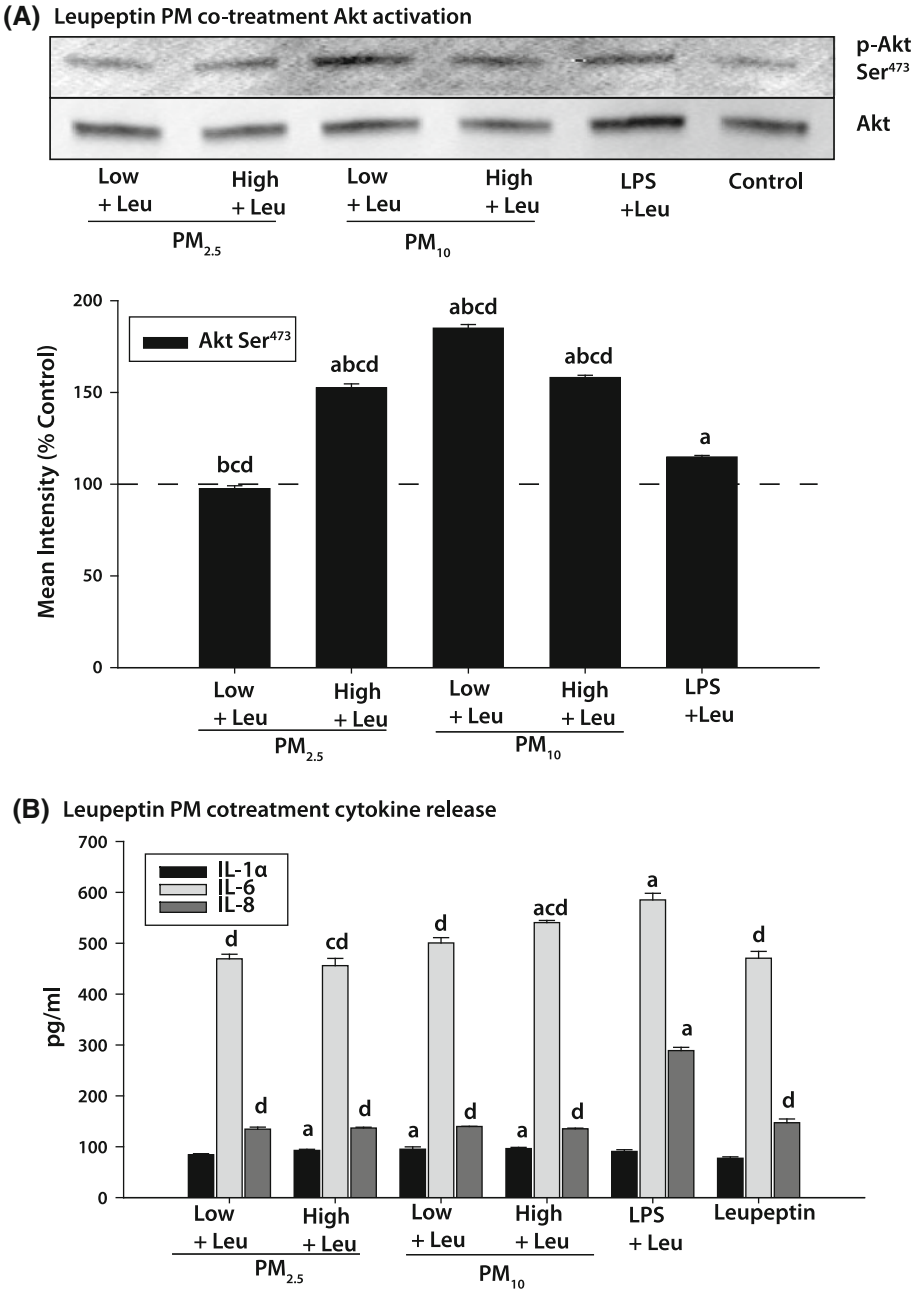

Fig. 8 Simultaneous leupeptin co-treatment alters PM-mediated Akt activity and cytokine release. **a** Co-incubation of the calpain inhibitor leupeptin with PM and LPS completely attenuated the ability to detect Akt phosphorylation at Thr^{308}, p-GSK, p-PTEN, and p-PDK as measured via western immunoblots. Only p-Akt (60 kDa) at Ser^{473} was detectable (compare with Figs. 1, 2). As phosphorylation of both sites on Akt is needed for full activity, it indicates that calpain is required for PM-mediated Akt activation. Western immunoblots were of cell lysates from BEAS-2B cells following 24-h co-exposures of 12.5 (low) and 25 (high) μg/ml concentrations of Cache Valley $PM_{2.5}$, PM_{10}, 10 ng/ml LPS plus 40 μg/ml of the calpain inhibitor leupeptin (leu) and leupeptin-only control. Gels were loaded with 10 μg/well. Figures are representative of a minimum of 3 independent experiments. **b** Calpain inhibition induced changes in PM-mediated cytokine release limiting the ability of most PM treatments, but not LPS to result in significantly elevated levels of IL-6 and IL-8 (compare with Fig. 6). *a* Significant from control, *b* significant from differing concentration of the same PM diameter, *c* significant from same concentration of differing PM diameter, *d* significant from LPS at $P \leq 0.05$

slightly different chemical profile generally present in differing PM diameter ranges (National Center for Environmental Assessment (Research Triangle Park N.C.) 2003). In any event, toxicological differences between size categories in vivo are mostly likely a result of differential deposition patterns in the respiratory tract, which are not possible to assess in an in vitro cell-based system. There were time-related differences between coarse and fine PM as evidenced by the 1- and 24-h exposures with pGSK, IκB, pPTEN, and p-Akt (Figs. 2, 3). More rapidly activated pathways may be stimulated by the components of fine PM and the slower by the coarse. Given that the longer 24-h exposure resulted in increased effects, individuals who have compromised clearance may be at greater risk for PM-mediated harm.

The USEPA states "Despite a strong consensus that exposure to PM induces adverse health effects […] relatively little is known about the specific physical or chemical characteristics of the particles that cause these effects or the mechanisms through which the adverse effects are induced" (NHEERL 2010). Thus, the present study may be helpful in revealing molecular and cellular mechanisms by which PM exerts harm.

This study demonstrates that ambient PM enhances activation of Akt in human pulmonary epithelial cells in vitro, and this activation is likely compensatory to ER

stress and other events. Activation of Akt involves PTEN inactivation and appears to depend upon calpain or other similar proteases, rather than receptor-mediated MyD88 activation. In total, these studies support the hypothesis that the pro-inflammatory effects of PM may be linked to Akt activation.

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